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SALT SENSITIVE HYPERTENSION AND OREXIN

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SALT SENSITIVE HYPERTENSION AND OREXIN

By

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Preface

The main body of this thesis is expected to be submitted to a peer review journal for publication and is a collaborative effort with co-authors Fengli Zhu, Dr. Robert A. Larson, Dr. Ningjun Li, Dr. Qing-Hui Chen, and Dr. Zhiying Shan. Author Contributions: Michael J. Huber, Fengli Zhu, Dr. Robert A. Larson, Dr. Ningjun Li, and Dr. Zhiying Shan performed experiments. Michael J. Huber, Fengli Zhu, Dr. Robert A. Larson, Dr. Ningjun Li, and Dr. Zhiying Shan analyzed data. Michael J. Huber, Dr. Ningjun Li, and Dr. Zhiying Shan prepared figures, Michael J. Huber drafted manuscript; Michael J. Huber, Dr. Qing-Hui Chen, and Dr. Zhiying Shan edited and revised manuscript; Michael J. Huber, Fengli Zhu, Dr. Robert A. Larson, Dr. Ningjun Li, Dr. Qing-Hui Chen, and Dr. Zhiying Shan approved the final version of manuscript; Michael J. Huber, Dr. Qing-Hui Chen, and Dr. Zhiying Shan conceptualized and designed the research.

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List of Abbreviations

AVP arginine vasopressin

SSHTN salt sensitive hypertension

PVN paraventricular nucleus

DS Dahl Salt Sensitive

OX1R orexin receptor 1

OX2R orexin receptor 2

ICV intracerebroventricular

NS normal salt

HS high salt

MAP mean arterial pressure

CO cardiac output

TPR total peripheral resistance

SV stroke volume

HR heart rate

NTS nucleus tractus solitarii

RVLM rostal ventrolateral medulla

IML intermediolateral cell column

CVLM caudal ventrolateral medulla

ECF extracellular fluid

ICF intracellular fluid

OVLT organum vasculosum of the lamina terminalis

SFO subfornical organ

MnPO median preoptic nucleus

CSF cerebrospinal fluid

SD Sprague Dawley

SNA sympathetic nerve activity

RSNA renal sympathetic nerve activity

SSNA splanchnic sympathetic nerve activity

Abstract

Elevated plasma arginine vasopressin (AVP) levels have been found in human hypertension subjects and several salt dependent experimental animal models of hypertension including the Dahl salt sensitive hypertension (SSHTN) model. The orexin system is involved in AVP regulation and its over activation has been implicated in hypertension, however, the role of brain orexin in SSHTN is unknown. We hypothesized that increased activity of orexin in the paraventricular nucleus (PVN), a prominent region in AVP production, contributes to SSHTN via enhancing AVP signaling. Eight-week-old male adult Dahl salt sensitive (DS) and age and sex matched Sprague Dawley (SD) rats were placed on either a high salt (HS, 8% NaCl) or normal salt (NS, 0.4% NaCl) diet for 5 weeks. Five weeks HS intake did not increase mean arterial pressure (MAP) or alter PVN mRNA expression of chronic neuronal activation marker Fra1, orexin receptor 1 (OX1R), or orexin receptor 2 (OX2R) but increased PVN AVP mRNA expression in SD rats. HS diet induced significant increases in MAP and PVN mRNA levels of Fra1, AVP, OX1R, and prepro orexin in DS rats. Intracerebroventricular (ICV) infusion of orexin A (0.2 nmol) increased PVN AVP mRNA levels in SD rats. Incubation of cultured hypothalamus neurons from newborn SD rats with orexin A resulted in increases in AVP mRNA expression which were attenuated by OX1R blockade. In addition increased cerebrospinal fluid (CSF) sodium concentration through ICV infusion of NaCl salt solution (4μmol) increased PVN OX1R and AVP mRNA levels in SD rats. Furthermore, bilateral PVN microinjection of the OX1R antagonist SB408124 resulted in a greater reduction in MAP in HS intake (-16±5 mmHg) compared to NS fed (-4±4 mmHg) anesthetized DS rats. These results suggest that elevated PVN OX1R activation may be involved in SSHTN through enhancing AVP signaling.

Chapter 1. Introduction

1.1 Overview and Significance

High blood pressure or hypertension is highly prevalent (102) and contributes significantly to both the incidence and mortality from cardiovascular disease in particular ischemic heart disease and stroke (80). Since cardiovascular disease is the leading global cause of death responsible for more than 17 million deaths per year (102) the discovery of the causes of hypertension are of great importance to society. It is well-established that elevated salt intake is a primary contributor to essential hypertension (74, 108, 148, 149) and the majority of individuals with essential hypertension are salt sensitive (148) meaning increased salt intake will increase blood pressure and decreased salt intake will decrease blood pressure in these individuals. Abnormalities in renal, vascular, hormonal, and neural responses to salt contribute to the pathogenesis of salt sensitive hypertension (SSHTN) (74). However, the cellular and molecular mechanisms that link elevated salt intake and hypertension are not fully understood. Therefore, the discovery of new mechanisms whereby salt contributes to hypertension may elucidate new targets for future treatment.

1.2 Regulation of Arterial Blood Pressure

As the blood is pumped by the heart it exerts a force over a unit surface area on the blood vessel walls that is termed blood pressure. With every single heartbeat, pressure on the vessel walls oscillates from a high pressure when the ventricles contract termed the systole portion of the heart cycle to a lower pressure when the ventricles relax and refill termed the diastole portion of the heart cycle. Therefore, when measured, usually at the brachial artery, blood pressure is given as two numbers, the pressure at systole over the pressure at

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The material contained in this chapter is in preparation for submission to a journal.

diastole. At any point in time during a single heart cycle the pressure will be somewhere in this range between the systole and diastole pressures. For adult's blood pressure should normally be <120/80 mmHg (14). Due to the pumping action of the heart, a pressure difference between the arteries and veins is created with pressure that is highest in the arteries and lowest in the veins. This pressure difference causes blood to flow in the circulation (92). Using the fundamental equation equivalent to Ohm's law for circuits that relates flow to resistance and pressure (∆Pressure=flow x resistance), and assuming pressure at the end of venous system to be negligible, then the mean pressure of the arterial side (MAP) of the systemic circulatory system can be calculated by the product of cardiac output (CO) and total peripheral resistance (TPR) (MAP=CO X TPR) (92). CO is further defined as the product of stroke volume (SV) and heart rate (HR) (CO=SV X HR) (118). Arterial blood pressure is regulated by two main systems, the kidneys regulation of blood volume and the autonomic nervous system control over the kidneys, heart, and vasculature.

1.2.1 Short Term Blood Pressure Regulation

Acute regulation of blood pressure (minutes to hours) is regulated via the baroreceptor reflex control of sympathetic nerve activity (SNA) (20). Mechanoreceptors located in the carotid sinus and aortic arch have discharge frequencies that depend on arterial pressure (112). Afferent signals are sent from these mechanoreceptors via the carotid sinus nerve (a branch of cranial nerve IX; glossopharyngeal) and aortic depressor nerve (a branch of cranial nerve X; vagus) to the nucleus tractus solitarii (NTS) in the medulla (114, 136). The NTS has excitatory projections to the caudal ventrolateral medulla (CVLM) (117) and the CVLM has inhibitory projections to the rostral ventrolateral medulla (RVLM) (114) which is the main nuclei responsible for tonic excitatory drive to sympathetic preganglionic neurons in the intermediolateral cell column (IML) of the spinal cord (54). When blood pressure is reduced the discharge frequencies of the mechanoreceptors in the carotid sinus and aortic arch are

reduced and the inhibitory influence of the CVLM on the RVLM is decreased. This results in an increase in sympathetic stimulation to the heart resulting in an increase in HR and force of contraction which increases SV and thus CO, increased sympathetic stimulation to blood vessels to increase vascular resistance, and activation of renal sympathetic nerves innervating the juxtaglomerular cells stimulating renin secretion and Ang II formation ultimately resulting in elevated blood volume and CO. Collectively these actions result in a compensatory increase in blood pressure (48). Alternatively, when blood pressure is increased the inhibitory influence of the CVLM on the RVLM is increased resulting in a decrease in sympathetic outflow to the heart, vasculature, and kidneys to decrease blood pressure (48) **(Figure 1.1)**.

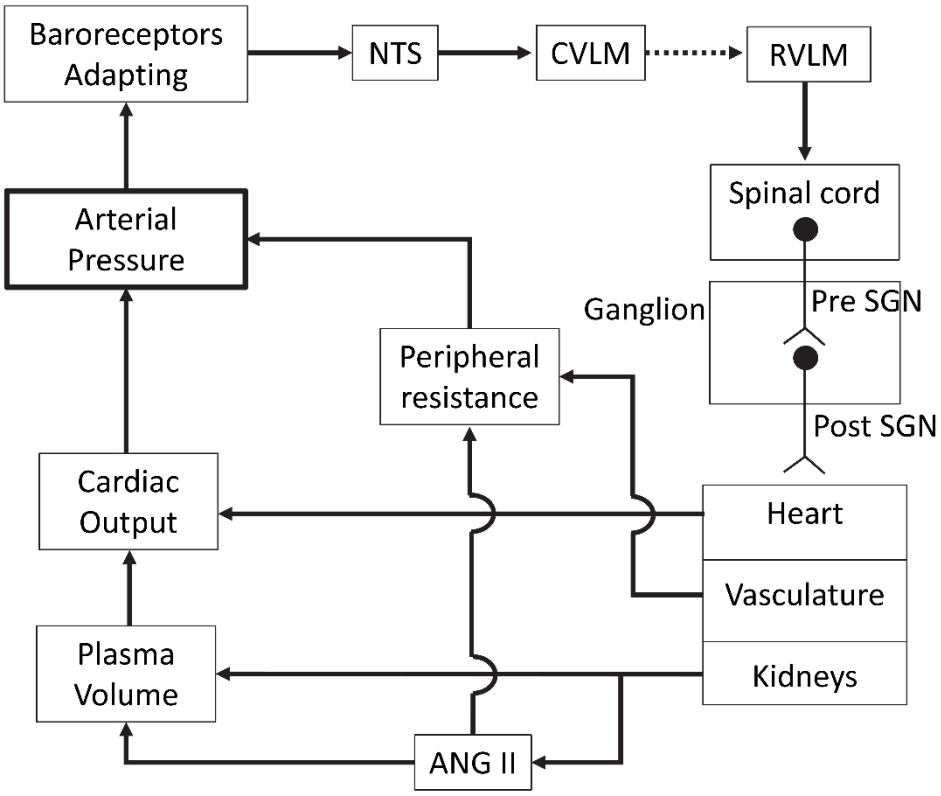

Figure 1.1 Diagram of the arterial baroreceptor reflex control of short term blood pressure regulation. Solid lines, excitatory; dashed line, inhibitory. (NTS) nucleus tractus solitarii, (CVLM) caudal ventrolateral medulla, (RVLM) rostral ventral lateral medulla, (SGN) sympathetic ganglionic neuron. Adapted from Brooks and Osborn (9).

1.2.2 Long Term Blood Pressure Regulation

Long term (weeks to months) regulation of arterial pressure is predominately regulated by the kidneys (57). The kidneys have the intrinsic ability to excrete salt (natriuresis) and water (diuresis) in response to increased perfusion pressure. Long term arterial pressure is set by the kidneys at a level that is required to excrete a volume of water or amount of salt equivalent to water or salt intake or in order to maintain sodium and water balance over an extended period of time (56). If salt intake or water intake is greater than excretion, extracellular fluid volume will increase which will increase blood volume leading to an increase in cardiac output and increase arterial pressure. In response to the increased pressure the kidneys will increase excretion of salt/water to reduce extracellular fluid volume and ultimately arterial pressure back to the equilibrium pressure were salt and water intake is equivalent to salt and water excretion. If salt or water intake is less than excretion, arterial pressure decreases and the kidneys decrease natriuresis /diuresis until arterial pressure returns to the equilibrium pressure (55, 56) **(Figure 1.2)**.

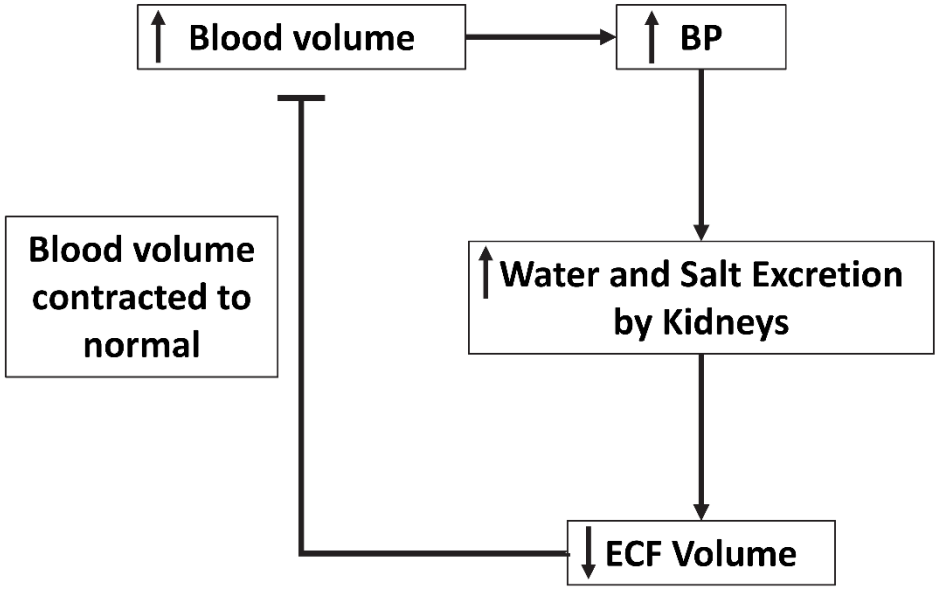

Figure 1.2 Diagram of the kidneys control of long term blood pressure regulation. (BP) blood pressure, (ECF) extracellular fluid.

1.3 Body Fluid Homeostasis and Vasopressin

Body water is separated into two compartments; the extracellular fluid (ECF) (which is comprised of interstitial fluid and plasma or intravascular fluid) and intracellular fluid (ICF) (3). Dissolved in the body fluids are many different salts including NaCl, KCl, and $NaHCO_3$. Out of these NaCl is consumed in greatest amounts and the cation Na+ is the major component of extracellular fluid (3). Therefore, its quantity largely determines extracellular osmolality (the concentration of all dissolved solutes in a solution) which determines extracellular and intracellular fluid volume (143). Changes in salt intake thus pose a common daily challenge to the maintenance of body fluid homeostasis (maintaining osmolality of body fluids and intravascular blood volume).

When NaCl is ingested its ion components Na+ and Cl- are absorbed in the small intestine and become evenly distributed in the plasma of the blood. This results in an increase in ECF osmolality (143). Since sodium ions do not easily cross the cell membrane they are confined to the ECF compartment. However, water can cross the cell membrane and moves from a region of low osmolality to high osmolality. Therefore, when sodium ions accumulate in the ECF compartment water moves from the ICF compartment (low osmolality) to the ECF compartment (high osmolality) were the fluid volume expands above normal volume (143). Several physiological responses are then initiated to restore sodium and water balance including increased thirst and release of arginine vasopressin (AVP) the antidiuretic hormone which increases renal reabsorption of water (3). Both compensatory responses dilute the extracellular Na+ concentration back to normal. Natriuresis via the kidneys, is also increased and the excess salt is eliminated in the urine and the ECF fluid volume naturally returns to normal (3).

If sodium concentration in the extracellular compartment is reduced ECF osmolality is reduced and water moves from the ECF compartment (low osmolality) to the ECF compartment (high osmolality) were fluid volume expands above normal (3). This results in a decrease of circulating sodium concentration and reduced blood pressure. The kidneys increase the release of

renin which catalyzes the formation of angiotensin I from angiotensinogen. The angiotensin converting enzyme catalyzes the reaction of angiotensin I to angiotensin II. Angiotensin II causes vasoconstriction in order to increase blood pressure and also stimulates the secretion of aldosterone from the adrenal gland. Aldosterone causes sodium reabsorption by the kidneys in order to prevent its loss in the urine (3). It has also been shown in animals that thirst along with AVP secretion are also responses to sodium depletion (17, 63) which supports that thirst and AVP secretion are additionally regulated by volume dependent mechanisms.

1.3.1 Neural Circuitry Involved in Osmotic Regulation of Vasopressin Release

AVP secretion is predominantly regulated by changes in blood osmolality and to a lesser extent by a decrease in ECF volume (43). In particular E.B. Verney was the first to show that only hypertonic solutions with solutes such as NaCl that are impermeable to the cell membrane and create osmotic pressure activate osmoreceptors that trigger thirst and AVP release (145). In this early study E.B. Verney concluded these osmoreceptors responsible for the regulation of AVP secretion were located in the brain. In a further study by Jewell et al. the location was narrowed down to be in the anterior hypothalamic area (67). It was latter suggested by two groups (95, 139) that these osmoreceptors likely lied outside of the blood brain barrier since carotid artery infusion of hyperosmolar solutions of NaCl, sucrose, or urea which do not pass the blood brain barrier effectively withdrew water and dehydrated the brain compartment however only hyperosmolar solutions of NaCl and sucrose which do not easily cross the cell membrane stimulated AVP release and thirst. Since urea easily penetrates the cell membrane it likely did not dehydrate and activate the supposed osmoreceptors that were present outside the blood brain barrier (95). Mckinley hypothesized the location of these osmoreceptors to be the two circumventricular organs of the lamina terminalis region which is located on the rostral wall of the 3rd ventricle that lack a blood brain barrier (95, 96). These circumventricular organs are called the organum vasculosum of the

lamina terminalis (OVLT) and the subfornical organ (SFO).

Most neurons in the brain are protected from changes in concentrations of circulating ions and hormones in the plasma that organs and tissues are usually exposed to by the presence of blood brain barrier that limits exchange between plasma in brain blood capillaries and brain tissue. However, the SFO and OVLT have a highly penetrable blood brain barrier due to fenestrated capillaries therefore they are sensitive to changes in plasma osmolality and circulating hormones (19). Another region of the lamina terminalis called the median preoptic nucleus (MnPO) lies between the OVLT and SFO. Both the SFO (82, 98) and OVLT (93) have direct excitatory projections to the hypothalamic paraventricular nucleus (PVN) as well as indirect excitatory projections to the PVN through projections to the MnPO (82, 98). The PVN is composed of parvocellular and magnocellular neurons. Parvocellular neurons have axon projections to the RVLM in the brainstem and IML in the spinal cord which play significant roles in regulating SNA (13, 116). Magnocellular PVN neurons synthesize AVP and transport it along nerve fibers that connect the hypothalamus with the posterior lobe of the pituitary gland called the neurohypophysis were it is stored and released (115).

The function of these circumventricular organs in the regulation of AVP secretion has been supported by lesion studies. Separate lesions of the OVLT and SFO and MNPO all attenuate osmotic stimulation of AVP release (86, 87, 94, 140). Total ablation of the lamina terminalis completely abolished osmotic stimulation of AVP (96) demonstrating all three regions may be important in osmotic stimulation of AVP release. In addition to these circumventricular organs as the brain site for osmotic regulation of AVP release, evidence suggests these regions are also responsible for angiontensin II regulation of AVP release. Cutting SFO efferents was found to attenuate angiotensin II induced stimulation of AVP release (73). It has also been shown that this region displays high density of the angiotensin II type 1 receptor (97).

1.3.2 Vasopressin Hemodynamics and Blood Pressure Control Mechanisms

In addition to the role of AVP in body fluid homeostasis, AVP is involved in blood pressure regulation. In normal conscious rats, dogs, and humans it has been found that acute 30-60 minute intravenous infusions of AVP exerts a significant dose dependent increase in MAP only at non physiological high plasma concentrations that are higher than those required for antidiuresis (26). However, it has also been found in rats, dogs, and humans that plasma AVP exerts vasoconstrictor activity at lower physiological concentrations within the range of maximum antidiuretic activity (26). The lack of a pressor response to increased plasma AVP levels at lower physiological concentrations is due to its action of decreasing cardiac output as acute 30-60 min Intravenous infusion of AVP exerts a dose dependent decrease in cardiac output in rats, dogs, and humans (26).

The ability of AVP to decrease cardiac output and buffer the pressor response may be explained by AVP activating reflex decreases in SNA. It has been shown that animals that have received baroreceptor denervation or where the central nervous system was totally eliminated develop an increased blood pressure response at lower amounts of intravenously administered AVP (22, 26, 110, 147). This enhancement of pressor sensitivity is greater than other vasoconstrictor agents such as angiotensin II or norepinephrine (22, 26, 110, 147) which demonstrates AVP is one of the most powerful circulating vasoconstrictors. One mechanism is the ability of circulating AVP to activate V1 receptors in the area postrema, which is another circumventricular organ lacking a blood brain barrier, and augment baroreflex sympatho-inhibition (60). Microinjection of AVP into the SFO decreased mean area under the curve blood pressure but not heart rate in SD rats which was blocked by V1 receptor blockade suggesting AVP may also activate V1 receptors in the SFO outside of the baroreflex neural circuitry resulting in a decrease in SNA (135).

The known mechanisms that circulating AVP can increase blood pressure include activation of V_{1A} $G_{q/11}$ protein coupled receptors on vascular

smooth muscle cells causing contraction, inducing vasoconstriction and increasing total peripheral resistance. The widely accepted calcium signaling pathway for the V_{1A} receptor on vascular smooth muscle cells involves phospholipase C activation of IP3 (8, 106). When activated the alpha subunit of the $G_{q/11}$ protein exchanges GDP for GTP and dissociates from the beta and gamma subunits. The GTP bound alpha subunit binds and activates phospholipase Cβ which causes the hydrolysis of $P1P_2$ increasing levels of DAG and IP_3. IP_3 binds the IP_3R on the endoplasmic reticulum and stimulates calcium release into the cell (8, 106) which causes contraction of the cell **(Figure 1.3)**. AVP may also act on the kidneys to increase water reabsorption causing an increase in intravascular volume which will increase SV and ultimately arterial pressure. This is mediated through activation of V_2 G_s protein coupled receptors on the basolateral membrane of the principle cells of the kidney collecting duct (8). When activated the alpha subunit of the G_s protein exchanges GDP for GTP and dissociates from the beta and gamma subunits. The GTP bound alpha subunit binds and activates adenylate cyclase which takes ATP and produces the second messenger cAMP. cAMP activates protein kinase A which increases exocytosis and decreases endocytosis of aquaporin water channel containing vesicles. This increases aquaporin 2 channels in the apical membrane and allows water to leave the collecting duct and enter the renal collecting duct cell where it eventually enters the blood (8) **(Figure 1.3)**.

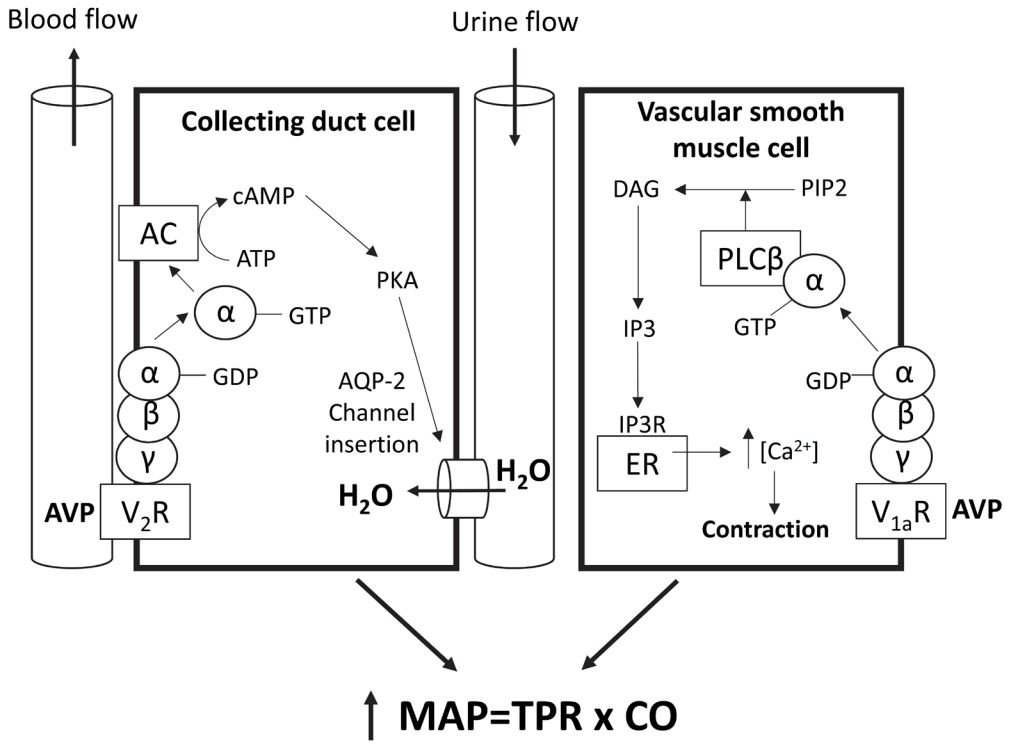

Figure 1.3 (left) Diagram of intracellular mechanism for vasopressin (AVP) binding V_2R (V_2 Receptor) on basolateral membrane of a kidney collecting duct cell increasing water reabsorption from the collecting duct which eventually enters the bloodstream increasing arterial blood pressure. (Right) Diagram of intracellular mechanism for AVP binding $V_{1a}R$ (V_{1a} receptor) on vascular smooth muscle cell increasing intracellular calcium and contraction leading to increased vascular resistance and arterial blood pressure. Abbreviations: (GDP) guanosine diphosphate, (GTP) guanosine triphosphate, (ATP) adenosine triphosphate, (cAMP) cyclic adenosine monophosphate, (AC) adenylyl cyclase, (PKA) protein kinase A, (AQP-2) aquaporin, (PLCβ) phospholipase Cβ, (PIP2) phosphatidylinositol (4,5)-bisphosphate, (DAG) diacylglycerol, (IP3) inositol (1,4,5)-triphosphate, (IP3R) inositol (1,4,5)-triphosphate receptor, (ER) endoplasmic reticulum, (MAP) mean arterial pressure, (TPR) total peripheral resistance, (CO) cardiac output. Adapted from Birnbaumer et al. (8).

1.4 Salt Sensitive Hypertension

The blood pressure values that define hypertension are a systolic blood pressure >140 mmHg or a diastolic blood pressure > 90 mmHg (14). Hypertension can be classified based on etiology. If the cause is not known it is termed essential or primary hypertension which accounts for most cases of

hypertension (10). Essential hypertension has the potential to be caused by a wide range of factors because blood pressure is influenced by genetics, behavior, and the environment (10). However, salt plays a central role in essential hypertension since it has been shown the majority of individuals with essential hypertension are salt sensitive (148) which suggests hypertension in these individuals is largely dependent on salt intake.

Arguably one of the most prominent figures in the discovery of the relationship between salt and hypertension is Dr. Lewis Dahl. Dahl was the first to compare salt intake and the prevalence of hypertension between several populations in the world. Using data from radiation research conducted by the United States Atomic Energy Commission after WWII, Dahl found that populations with higher salt consumption had a higher prevalence of hypertension (32). Along with Dr. Dole and Cotzias, Dahl was also the first to confirm in the 1950's through human metabolic studies that the reduction in blood pressure from Kempner's rice-fruit diet, that had been previously found to be useful for treatment of hypertension (70, 71), was due to its low sodium content (40-42). Dahl later hypothesized that since a low salt diet lowered blood pressure a high salt (HS) diet may cause an increase in blood pressure (33). Dahl tested this hypothesis through a series of studies using an experimental form of hypertension were Sprague-Dawley rats were fed a chronic HS diet while their blood pressure was monitored. He found that some rats had no blood pressure response and the rest of the rats developed varying degrees of elevations in blood pressure (30, 31, 34). From these experiments it was apparent to Dahl that genetic factors that determined susceptibility to salt were responsible for the variations in blood pressure responses (37). This led to development of the Dahl rat model of SSHTN.

1.4.1 Dahl Salt Sensitive Hypertension Model

In the 1960's Dr. Lewis Dahl developed his animal model of SSHTN that is still one of the most widely studied models today. Dahl fed Sprague-Dawley (SD) rats a L-triiodothyronine-sea salt diet then selected and bred the rats that developed the greatest elevations in blood pressure. He also selected and bred

rats that had no blood pressure response to the HS diet. Through repeated diet treatment, selection, and inbreeding of both groups Dahl obtained entire statistically different populations of rats that were very sensitive (Dahl salt-sensitive rats (DS) and the other that was very resistant called (Dahl salt resistant rats) to the development of HS induced hypertension (35-37). This ability to isolate separate populations confirmed that the variability in blood pressure in response to a high salt diet in the original unselected population of SD rats was due to genetic factors that controlled sensitivity to salt rather than due to statistical extremes of a homogenous population (37).

1.4.2 Vasopressin in Hypertension and Contribution to Dahl Salt Sensitive Hypertension Model

Increased osmolality naturally results from hypernatremia and activates osmoreceptors resulting in AVP release. Given AVP plays a role in blood pressure regulation, It is logical that alterations in this essential physiological response may therefore be a mechanism by which a HS diet can lead to the development or maintenance of hypertension. However, In normal rats and dogs long term administration of AVP to increase levels observed in hypertension does not produced sustained hypertension (111, 134). This has been demonstrated in dogs were AVP was infused for 3-4 weeks to increase plasma levels to those observed in hypertension where water intake was regulated to remain constant (134). MAP increased around 40 mmHg along with an increase in body weight followed by a return of MAP and body weight towards normal at the end of the two weeks. However, in dogs where total body fluid volume was servo controlled MAP or body weight did not change over a two-week period were the same amount of AVP was infused (21). This finding supports the notion that the antidiuretic actions of AVP may be more important than the vasoconstrictive actions of AVP in hypertension although additionally AVP may not sustain hypertension in normal animals because it cannot sustain blood volume expansion (25).

This phenomenon where the antidiuretic actions of AVP do not last is called AVP escape. One mechanism of AVP escape is simply the kidneys

intrinsic ability of pressure diuresis in response to elevated circulating AVP causing blood volume expansion and elevated renal perfusion pressure since long term administration of a subpressor dose of AVP in dogs with only 30% of normal renal mass to reduce natriuresis and diuresis ability induced sustained hypertension for 14 days (88). Blood pressure significantly increased within three days of AVP administration and decreased back to normal within three days when administration of AVP was stopped. Other mechanisms of AVP escape include down regulation of V2 receptors in the inner medulla of the kidneys (141), decreased expression of aquaporin-2 water channel in the renal collecting duct (44), and Increased renal prostaglandin E2 (51). Also AVP may not sustain hypertension due to its opposing actions on controlling blood flow to the renal medulla resulting in its inability to chronically reduce renal medullary blood flow. V1 receptor activation presumably on the medullary vasa recta decreases renal medulla blood flow while V2 receptor activation increases renal medulla blood flow (104). In conscious rats, chronic renal interstitial infusion of an V1 receptor agonist significantly decreased inner medullary blood flow, produced no change in cortical blood flow, and significantly increased MAP. However chronic renal interstitial infusion of AVP which would activate V1 and V2 receptors did not significantly decrease inner medulla blood flow or significantly increase MAP (23). Evidence suggests V2 receptor activation in the inner medulla collecting duct cells stimulates nitric oxide production which may oppose V1 receptor action and increase medullary blood flow by vasodilation of the medullary vasa recta (107).

Nevertheless enhanced AVP levels have been found in human hypertension subjects (24, 109) and several animal models of experimental hypertension such as spontaneously hypertensive rats (28, 99), partial nephrectomy-salt hypertension (77), deoxycorticosterone-salt (DOC-salt) hypertension (29), and renovascular hypertension (100). DS rats fed a HS diet have greater plasma AVP levels compared to Dahl salt resistant rats fed a HS diet (7, 90, 124, 146). However, the contribution of AVP to the Dahl model of SSHTN is still controversial, as studies involving the administration of AVP

antagonists to DS rats with salt induced hypertension have produced varying results. Acute intravenous administration of the V_{1a} receptor antagonist $(CH_2)_5$ VDAVP had no effect on the MAP of hypertensive DS rats fed a HS diet (90). Oral administration of the V_{1a} receptor antagonist OPC-21,268 prevented any further increase in systolic blood pressure for the first 6 days of treatment in male DS rats that were placed on an 8% NaCl HS diet 10 days prior (27). However, this effect was transient and systolic blood pressure increased to levels below control rats but not significantly different at the end of 12 days with treatment of OPC-21,268. A More recent study showed that administration of a dual AVP receptor antagonist RWJ-676070 produced a transient decrease in MAP in hypertensive DS rats on a HS diet (53). These studies suggest AVP contributes to HS induced hypertension in DS rats however hypertension in this model is not completely dependent on AVP and other mechanisms contribute as well. Interestingly chronic intravenous infusion of a subpressor dose of AVP in DS rats significantly increased MAP after 1 day and after 14 days increased MAP around 18 mmHg. Brown Norway rats that received the same treatment did not show any change in MAP throughout the 14 days (150). This data demonstrates DS rats are very sensitive to AVP. It has been hypothesized by Gunnet et al. (53) that this enhanced sensitivity to AVP may also explain the transient blood pressure reduction effects by AVP receptor antagonists in HS fed DS rats.

In addition to the consequences elevated AVP levels may have on V_{1a} activation leading to increased vascular resistance, elevated AVP levels could possibly contribute to Dahl SSHTN hypertension due to volume expansion. It has been demonstrated that the development of hypertension in DS rats in response to HS diet is volume dependent. Servo controlled prevention of blood volume expansion prevented the rise of MAP in DS rats in response to HS intake (20 mEq/ day sodium) suggesting blood volume expansion may be an important factor in the BP development in the Dahl model (50). This is consistent with the hemodynamics of DS rats on a 8% NaCl HS diet. After 4 weeks on a 8% NaCl HS diet it has been shown hypertension is mainly

developed and maintained due to blood volume expansion causing increased CO. At 8 weeks on an 8% NaCl HS diet CO is reduced (most likely due to autoregulation to maintain consistent tissue flow) and hypertension is maintained due to increased TPR (133). However, vasopressin has not been directly linked to this blood volume expansion stage of hypertension in DS rats. Despite the lack of evidence thus far that convincingly shows AVP contributes the Dahl model of SSHTN, the central mechanisms that mediate enhanced AVP levels found in this model are not well known.

1.5 Orexin

The neuropeptides orexin A and orexin B (also known as hypocretin 1 and hypocretin 2) were first discovered independently in 1998 by two different groups. Sakurai et al. identified two neuropeptides isolated from rat brain tissue that were produced from the same precursor polypeptide by proteolytic processing (120). They found that both peptides were ligands of two closely related G protein-coupled receptors that had no previously known ligands. When centrally administered in rats, both peptides stimulated increased food consumption and the precursor polypetide mRNA was upregulated in 48 hour fasted rats. Therefore, they called these peptide ligands orexins (orexin A and orexin B) similar to the greek word orexis meaning appetite, and the G protein-coupled receptors they activated orexin receptor 1 (OX1R) and orexin receptor 2 (OX2R). They termed the precursor polypeptide prepro-orexin and the messenger mRNA that encoded it prepro-orexin mRNA (120). Sakurai et al. was the first to describe in detail the structure of the orexins produced from prepro-orexin polypeptide. Orexin A is a 33 amino acid peptide with two intrachain disulfide bonds while orexin B is a linear 28 amino acid peptide (120). They also found that orexin A and orexin B bind OX2R with equal affinity while orexin A binds to the OX1R with a higher affinity than orexin B (120).

Independently de Lecea et al. identified the mRNA for the same precursor peptide and predicted this peptide was cleaved to form a pair of peptides (39). They called these peptides hypocretin 1 and hypocretin 2 because their amino acid sequence was similar to members of the hormone

family the incretins and because they found the messenger mRNA and precursor polypeptide to be located in cell bodies of neurons in the hypothalamus (39).

Soon after the discovery of orexin subsequent major immunohistochemistry studies examined where orexin producing neurons were localized in the brain and the regions of the brain that these neurons projected to. The cell bodies of neurons containing orexins were found to be exclusively localized only in the hypothalamus particularly in the lateral hypothalamic area, perifornical nucleus, dorsomedial hypothalamic nucleus, dorsal hypothalamic area, and posterior hypothalamic area (38, 105, 113) in the rat brain. However, these orexin containing neurons have widespread projections throughout the entire brain including the cerebral cortex, thalamus, circumventricular organs, limbic system, and brainstem as well as throughout the hypothalamus (38, 105, 113). Consistent with this extensive projection throughout the brain orexin A and orexin B are reported to function as excitatory neurotransmitters (39, 72, 138) and regulate numerous physiological processes including sleep and arousal (11, 138), feeding behavior (45, 61, 66, 120), emotion (68, 76), reward (59), and energy homeostasis (101) processes. Accumulation of studies now suggest the orexin system serves an overall function of receiving internal and external environment information and serves to regulate arousal and autonomic function to support motivated behaviors (119).

1.5.1 Orexin Control of Cardiovascular Function

Studies suggest one of the autonomic functions the orexin system regulates is the central nervous system control of cardiovascular function. Prepro-orexin knockout mice exhibit a lower basal arterial pressure in both anesthetized and conscious conditions (69). Also rats with genetic ablation of orexin neurons have lower systolic and diastolic pressure in both sleep and wake states (123). Central administration of orexin A and orexin B increases HR, MAP, and SNA in conscious (84, 91, 121, 129, 131) and anesthetized animals (4, 12, 125, 126) indicating one mechanism orexin A regulates circulation and blood pressure is through regulating SNA. The orexin system

has also been implicated in AVP regulation. ICV administration of orexin A increases AVP mRNA in parvocellular neurons of the PVN in conscious rats (1) and also plasma AVP levels in conscious rabbits (91). These findings are consistent with orexin containing nerve terminals (6, 15, 16, 105, 113, 126, 151) and receptors from *in-situ* hybridization studies (83, 89, 144) and immunohistochemistry studies (5, 18, 62) residing in many cardiovascular control regions of the brain including both magnocellular and parvocellular neurons of the PVN. Studies focusing on the action of orexin in the PVN have shown that orexin-A has excitatory actions by depolarizing both magnocellular and parvocellular neurons (47, 122, 130). The actions of orexin-A on magnocellular and parvocellular PVN neurons based on current evidence are shown in **figure 1.4**.

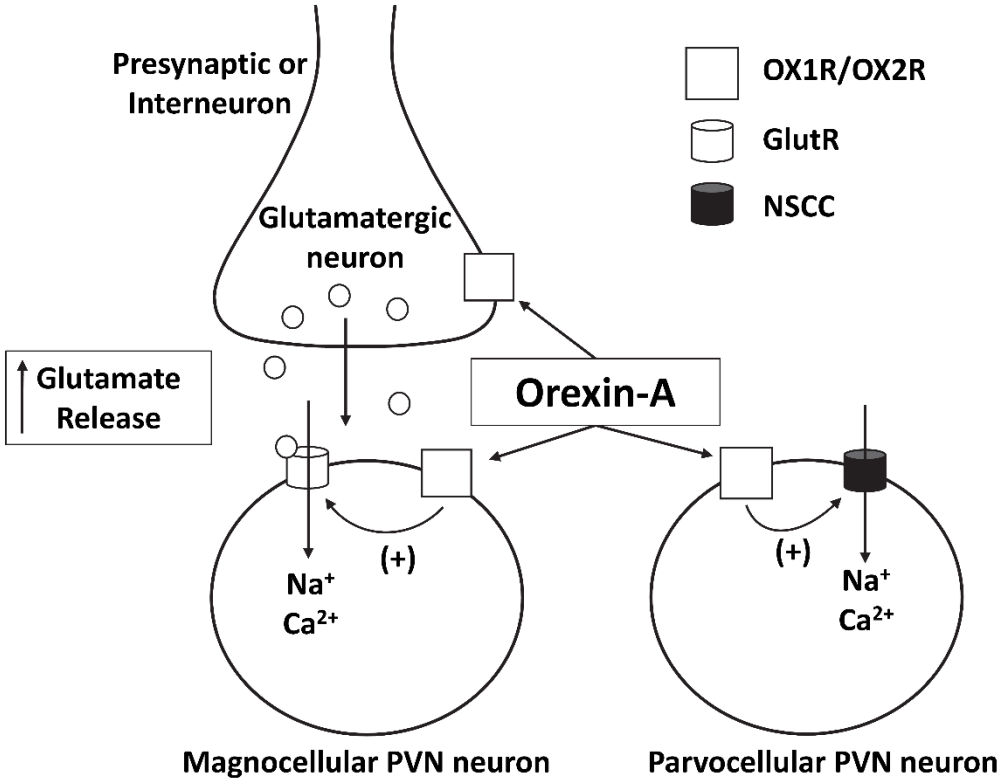

Figure 1.4 Orexin-A depolarizes magnocellular and parvocellular paraventricular nucleus (PVN) neurons. Orexin-A may activate either glutamate containing presynaptic or interneurons increasing glutamate release to magnocellular PVN neurons activating postsynaptic glutamate receptors

(GlutR). Orexin-A may also enhance postsynaptic glutamate receptor function in magnocellular PVN neurons. Orexin-A depolarizes parvocellular PVN neurons through activation of non-selective cation channels (NSCC). Abbreviations: orexin receptor 1 (OX1R), orexin receptor 2 (OX2R). Figure based on data from Follwell and Ferguson (47).

1.5.2 Orexin System Contributes to Hypertension

Accumulating evidence indicates that hyperactivity of the orexin system contributes to several animal models of hypertension. Oral administration of the dual orexin receptor antagonist almorexant significantly decreased systolic blood pressure, diastolic blood pressure, MAP, and HR compared to pre treatment in conscious spontaneously hypertensive rats in both wakefulness and sleep during dark and light periods of the diurnal cycle (81). However, the same dose of the dual orexin receptor antagonist did not affect blood pressure or HR in Wistar Kyoto rats (81). This study suggests that over activation of the orexin system contributes to the maintenance of hypertension in the SHR hypertension model. Furthermore, ICV administration of an OX2R antibody or antagonist and RVLM injection of an OX2R antagonist results in a significant reduction in MAP and HR in spontaneously hypertensive rats but not in Wistar Kyoto rats (78). This study suggests that elevated OX2R activity in the RVLM contributes to hypertension in the spontaneously hypertensive rat model.

The orexin system has also recently been implicated in the obese Zucker rat model of obesity-related hypertension. Microinjection of the OX1R antagonist SB334867 into the PVN significantly decreased MAP and renal sympathetic nerve activity (RSNA) in obese Zucker rats but not in lean Zucker rats (152). PVN OX2R blockade did not affect MAP or RSNA in obese Zucker rats or lean Zucker rats. PVN OX1R protein levels were significantly greater in 15 week old obese Zucker rats compared to lean Zucker rats (152). Additionally orexin A induced a greater increase in firing rate of spinally projecting PVN neurons in obese Zucker rats compared to lean Zucker rats which was attenuated by OX1R antagonist SB334867 but not OX2R antagonist TCS OX2 29 (152). This study suggests that upregulation of the OX1R contributes to

enhanced neuronal activity of PVN neurons and contributes to augmented SNA and hypertension in the obese Zucker rat model of obesity related hypertension. However, the role orexin plays in SSHTN is unknown.

1.6 Summary and Hypothesis

AVP production and release is a homeostatic response to hyperosmolality following salt ingestion and is involved in blood pressure control. It has been demonstrated that enhanced AVP levels associated with DS rats on a HS diet may contribute to Dahl SSHTN. However, the central mechanisms were HS stimulation may increase AVP levels and contribute to SSHTN are not well known. Based on recent evidence that increased activity of the brain orexin system is involved in hypertension we sought to investigate its involvement in SSHTN. Previous studies on the orexin systems role in hypertension have focused on orexin mediated changes in SNA therefore, we intended to focus on orexin mediated changes in AVP signaling. Taking into account the importance of the PVN in AVP and blood pressure control, and that orexin is involved in the regulation of PVN neuronal activity and AVP regulation we hypothesized that increased activity of the orexin system in the PVN may be responsible for enhanced AVP levels in HS fed DS rats.

The link between how increased salt intake could activate the orexin system is unclear at the present time. There is no direct evidence that orexinergic neurons are osmosensitive or receive input from osmoreceptors in the lamina terminalis. However, it has previously been shown that prepro-orexin mRNA is upregulated In the hypothalamus of 48 hour water deprived rats (75) indicating orexin producing neurons may be sensitive to changes in osmolality. Additionally, the lamina terminalis has been shown to have projections to the lateral hypothalamus (58, 65), a region where the cell bodies of orexin producing neurons are found.

In DS rats a combination of genetic abnormalities may lead to chronic activation of the orexin system in the PVN in response to elevated salt intake. It has been demonstrated that DS rats have genetic functional abnormalities of the kidneys that reduced natriuresis or the ability to excrete salt (85). It has

been shown that in response sodium is abnormally sequestered in the CSF (132). Specifically, it has been shown that the CSF sodium concentration of DS rats increased to 6mM after 6 days on an 8% HS diet before development of hypertension hypertension (64). It has been previously hypothesized that CSF sodium concentration is increased in response to sodium retention in DS rats (132). Abnormal transport across the choroid plexus (2) may be responsible for increased CSF sodium concentration in DS rats.

Given this evidence in DS rats, it is plausible that in response to a HS diet reduced ability of the kidneys to excrete salt may cause cerebrospinal fluid (CSF) sodium concentration to increase as hypothesized by Simchon et al. (132). The circumventricular organs in the lamina terminalis may sense increased osmolality due to elevated CSF sodium concentration which may subsequently activate orexin producing neurons located in the hypothalamus. Alternatively increased CSF sodium concentration may directly activate orexin producing neurons. This may result in increased orexin release to the PVN which could potentially increase PVN neuronal activity resulting in enhanced AVP secretion. Elevated plasma AVP levels may then play a role in the Dahl model of SSHTN through its vasoconstrictive or antidiuretic actions. In addition, the reduced ability of natriuresis by the kidneys of DS rats may support chronic activation of the orexin system and may explain why orexin producing neurons would not be chronically activated in SD or Dahl salt resistance rats in response to a high salt diet. This hypothesis is shown in **figure 1.5**.

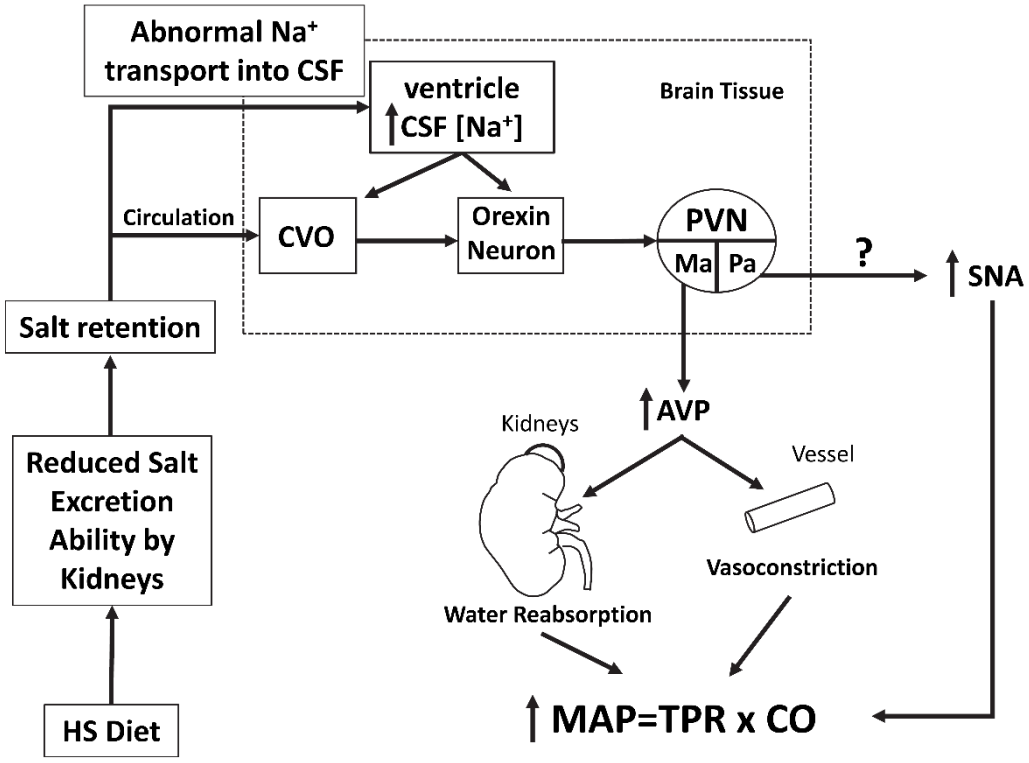

Figure 1.5 Hypothesis for high salt (HS) induced activation of the orexin system in the paraventricular nucleus (PVN) leading to enhanced vasopressin (AVP) levels in Dahl salt sensitive rats. A HS diet may increase cerebrospinal fluid (CSF) fluid sodium concentration. Circumventricular organs (CVO) in the lamina terminalis may sense increased osmolality and activate orexin producing neurons. This may increase orexin release to the paraventricular nucleus and activate magnocellular (Ma) neurons and increase release of vasopressin (AVP) into the blood. Elevated blood AVP levels may contribute to hypertension in this model through vasoconstriction or increased water reabsorption. Reduced ability of natriuresis by the kidneys of Dahl salt sensitive rats may support chronic activation of the orexin system and may explain why orexin would not be chronically activated in Sprague Dawley or Dahl salt resistance rats in response to a high salt diet. Whether parvocellular (Pa) pre sympathetic neurons are activated by orexin and result in increased sympathetic nerve activity (SNA) contributing to hypertension in this model remains to be determined. (MAP) mean arterial pressure, (TPR) total peripheral resistance, (CO) cardiac output.

Chapter 2. Methods

2.1 Animals

All rats used in this study were purchased from Charles River Laboratories (Wilmington, MA). Eight-week-old male adult DS and age and sex matched SD rats were placed on either a normal salt (NS), 0.4% NaCl diet or HS 8% NaCl diet (Envigo RMS, IN, USA) for 5 weeks, and used for blood pressure measurement, brain mRNA measurement, and sympathetic nerve activity recording. Adult male SD rats (350-500 g) were used for brain intracerebroventricular (ICV) infusion. Cultured hypothalamic neurons were obtained from newborn SD rats. All animals were housed in two rats per cage and kept on a 12:12-h light-dark cycle in a climate-controlled room. Rat chow and water were provided ad libitum. All of the animal experiments followed protocols that were approved by the Michigan Technological University Institutional Animal Care and Use Committee.

2.2 Blood Pressure Measurement

Prior to diet treatment eight week old male adult DS rats were anesthetized with isoflurane (3%) and telemetry transmitters were implanted into the abdominal aorta, and arterial pressure was monitored as described previously (128). Blood pressure in age and sex matched SD rats was monitored during diet treatment via volume pressure tail cuff method.

2.3 Real-time PCR Analysis of PVN Fra1, AVP, Prepro Orexin, OX1R, and OX2R mRNA Expression

Eight-week-old male adult DS rats and age and sex matched SD rats were placed on an either a normal salt (NS), 0.4% NaCl diet or HS 8% NaCl diet for 5 weeks. After diet treatment animals were euthanized, brains were removed and PVN tissue was punched. Punched PVN tissues were subjected to RNA isolation, using RNeasy plus Mini kit (Qiagen, CA, USA) following the manufacturer's instructions.

The material contained in this chapter is in preparation for submission to a journal.

Real-time PCR was performed to measure mRNA levels of Fra1, AVP, prepro orexin, OX1R, and OX2R using the specific primer and probe (Applied Biosystems, Foster City, CA) in the Step One Plus Real Time PCR System (Applied Biosystems). Data were normalized to GAPDH mRNA.

2.4 Preparation of Neuronal Cultures

Primary neuronal cultures were made from the hypothalamus containing the PVN of 1-day-old SD rats as described previously (127). Briefly, rats were euthanized and the hypothalamus was immediately dissected and tissues were combined. Neurons were dissociated from each other and plated in poly-L-lysine culture dishes. Neuronal cultures contained >95% neurons (remaining cells were primarily astroglia). The cultures were maintained for 10–14 days before their use in experiments.

2.5 Measurement of Orexin A Effect on AVP Expression in Cultured Brain Neurons

Primary neuronal cultures from the hypothalamus of 1-day old SD rats were incubated with vehicle control or differing concentrations of orexin (10nM, 100nM, 1μM, 10μM) for 6 hours. The cells were collected, RNA was isolated, and real-time PCR was performed to measure mRNA levels of AVP. We then coincubated neuronal cultures with 1μM orexin with or without the orexin receptor 1 antagonist (OX1Ra) SB-408124 (100 μM) or orexin receptor 2 antagonist (OX2Ra) TCS OX2 29 (100 μM) (Tocris Bioscience, united, Kingdom) for 6 hours. The mRNA level of AVP was determined by quantitative real-time PCR as described in the methods. Each set of experiments was performed using three separate culture dishes, and all cDNA samples were assayed in duplicate. The whole experiment was repeated three times. Data were normalized to GAPDH mRNA.

2.6 Intracerebroventricular (ICV) Injections

Adult male SD rats (350-500 g) were anesthetized with isoflurane (3% in O_2). Body temperature was held at 37°C with a water-circulating pad. Animals were placed in a stereotaxic head frame, and the skull was leveled between bregma and lambda. A small piece of skull was removed so that a Hamilton

syringe could be lowered vertically into the left lateral ventricle. The stereotaxic coordinates for I.C.V injection into the left lateral ventricle were: 0.8–0.9 mm caudal to bregma; 1.4–1.8 mm lateral to midline; and 3.2–3.8 mm ventral to dura. Each rat received only one injection. For I.C.V injection, vehicle control (0.9% saline) was injected at a fixed volume of 2 µl over 2 min., orexin A (.2nmol) was injected at a fixed volume of 4 µl over 5 min., and hypertonic saline (4 µmol) was injected at a fixed volume of 2 µl over 2 min. Orexin A (Sigma-Aldrich, St. MO, USA) was dissolved in 0.9% saline. Drugs were injected into the left lateral ventricle using a UltraMicroPump3 (WPI). Three hours following ICV injection, rats were euthanized and brain PVN tissues were punched out and received real time PCR analysis for AVP mRNA expression as described in this section.

2.7 Experimental Preparation for Sympathetic Nerve Activity Recordings

Animal surgery was performed following the previously described protocol (52). Briefly, rats were anesthetized with an intraperitoneal injection containing a mixture of α-chloralose (80 mg/kg) and urethane (800 mg/kg). Adequate depth of anesthesia was assessed before surgery by the absence of pedal and corneal reflexes and by failure to withdraw the hindlimb in response to pinching the paw. Animals were instrumented with an arterial catheter inserted into the aorta through a femoral artery. The catheter was connected to a pressure transducer to measure arterial blood pressure (ABP). Heart rate (HR) was obtained from the R-wave of the electrocardiogram (ECG) (lead I). A catheter was also placed in the left femoral vein to administer drugs. After tracheal cannulation, rats were paralyzed with gallamine triethiodide (25 mg·kg−1·h−1 iv) and artificially ventilated with oxygen-enriched room air. After paralysis, adequate depth of anesthesia was determined by lack of pressor responses to noxious foot pinch. Supplemental doses of anesthesia equal to 10% of the initial dose were given when needed. End-tidal P_{CO2} was continuously monitored and maintained within normal limits (35–40 mmHg) by adjusting ventilation rate (80–100 breaths/min) and/or tidal volume (2.0–3.0 ml).

Body temperature was held at 37°C with a water-circulating pad.

2.8 Sympathetic Nerve Activity (SNA) Recording

SNA recording was performed as previously described (52, 137). With the use of a left flank incision, a left renal and splanchnic sympathetic nerve bundle was isolated from surrounding tissue and mounted on a stainless steel wire electrode (0.127-mm OD; A-M Systems), and covered with a silicon-based impression material (Coltene, Light Body) to insulate the recording from body fluids. The recorded signal was directed to an AC amplifier (P511; Grass Technologies) equipped with half-amplitude filters (band pass: 100-1,000 Hz) and a 60-Hz notch filter. The processed signal was rectified, integrated (10-ms time constant), and digitized at a frequency of 5,000 Hz using a 1401 Micro3 analog-to-digital converter and Spike 2 software (7.04 version; Cambridge Electronic Design, Cambridge, UK). The background noise was determined by a bolus injection of hexamethonium (30 mg/kg iv), a ganglionic blocker, at the end of the experiment and was subtracted from all the integrated values of SNA.

2.9 Paraventricular Nucleus (PVN) Injections

PVN injections were performed as previously described (52). Animals were placed in a stereotaxic head frame, and the skull was leveled between bregma and lambda for PVN injections. A small piece of skull was removed so that a single-barreled glass microinjector pipette could be lowered vertically into the PVN. The stereotaxic coordinates for PVN injections were the following: 1.2–1.6 mm caudal to bregma; 0.5–0.7 mm lateral to midline; and 7.0–7.4 mm ventral to dura. The OX1R antagonist SB408124 (Santa Cruz Biotechnology, Texas, USA) was dissolved in DMSO. SB408124 (30pmol) was microinjected into the PVN bilaterally in a volume of 50 nl per side with a pneumatic pump (WPI). The approximate interval between two bilateral injections was ~5 min. The volume of each injection was determined by measuring the movement of the fluid meniscus within the microinjector pipette using a dissecting microscope equipped with an eyepiece reticule. At the end of each experiment,

Chicago blue dye solution (2% in saline, 50 nl) was injected into the PVN to mark the site of each injection. Brains were removed and post fixed for 5 days at room temperature in 4% paraformaldehyde. Brain coronal sections containing the PVN were cut, and microinjection sites were identified under light microscopy. Rats with injection site(s) not inside the PVN were excluded from data analysis.

2.10 Data analysis

Summary data are expressed as means ± SEM. For SNA recording splanchnic sympathetic nerve activity (SSNA) and RSNA were determined as an average of the rectified, integrated signal. Baseline values of all recorded variables were obtained by averaging a 10-min segment of data recorded immediately before PVN microinjection in anesthetized rats. SSNA, RSNA, and MAP responses to OX1Ra SB408124 were obtained by averaging a 2-min period centered on the maximal response. Data are presented as percent (%) change from baseline after subtracting background noise determined with bolus injection of the ganglionic blocker hexamethonium (30 mg/kg). Both in vivo and in vitro data were analyzed using either a one-way anova or unpaired student t-test. Post hoc analysis was performed with Newman-Keuls multiple comparison test. Differences were considered statistically significant at a critical value of $P < 0.05$.

Chapter 3. Results

3.1 High Salt Diet Increases Blood Pressure in DS Rats

In order to confirm a HS diet induces hypertension in DS but not SD rats, eight-week-old male adult DS rats and age and sex matched SD rats were placed on either a NS, 0.4% NaCl diet or HS 8% NaCl diet for 5 weeks. Blood pressure was monitored via telemetry transducer. SD rats on a HS diet showed no change in MAP compared to NS diet SD rats (HS: 108±2 vs NS: 105 ± 1 mmHg). DS rats on a HS diet had significantly ($P<0.05$) increased MAP (HS: 159 ± 6 vs. NS: 111 ± 1 mmHg) compared to NS diet DS rats **(Figure 3.1)**.

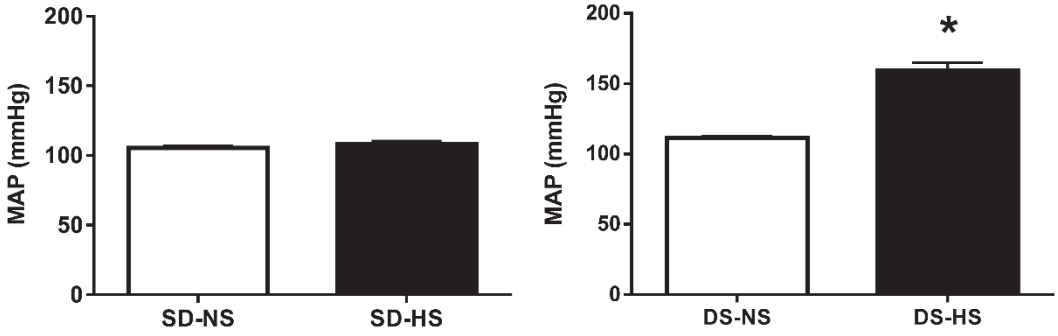

Figure 3.1 Conscious mean arterial pressure (MAP) for Sprague Dawley (SD) rats and Dahl salt sensitive rats (DS) after 5 weeks on a normal salt (NS; 0.4% NaCl) and high salt (HS; 8% NaCl) diet.

3.2 High Salt Diet Increases PVN mRNA levels of Fra1, AVP, OX1R, and Prepro Orexin in DS Rats

Next, we sought to determine if a HS diet alters PVN AVP and orexin system component expression in SD and DS rats. Eight-week-old male DS rats

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The material contained in this chapter is in preparation for submission to a journal.

and age and sex matched SD rats were divided into two groups and were fed either a NS (0.4%) or HS (8% NaCl) diet for 4-6 weeks.The rats were then euthanized, PVN tissue was punched out and real time PCR was performed to assay the chronic neuronal activation marker Fra1, AVP, prepro orexin, OX1R, and OX2R mRNA levels. SD rats on a HS diet showed no significant change in PVN mRNA levels of Fra1 (HS: 1.46±0.37 vs. NS:1.0±0.18 fold; n=4-6; P>0.05), OX1R (HS: 1.21±0.06 vs. NS:1.0±0.13 fold; n=4-6; P>0.05), or OX2R (HS: 0.82±0.05 vs. NS:1.0±0.17 fold; n=4-6; P>0.05), compared to NS diet SD rats. SD rats on HS intake showed significantly increased PVN mRNA levels of AVP (HS: 1.52±0.09 vs. NS:1.0±0.09 fold; n=3-6; P<0.05), compared to NS intake SD rats **(Figure 3.2)**. HS intake induced significant increases in PVN mRNA levels of Fra1(HS: 2.4±0.48 vs. NS:0.93±0.13; n=6; P<0.05), AVP (HS: 2.4±0.46 vs. NS: 1.0±0.09; n=5-6; P<0.05), prepro orexin (HS: 2.5±0.46 vs. NS: 1.0±0.28; n=7; P<0.05), and OX1R (HS: 1.5±0.09 vs. NS: 1.0±0.09; n=7; P<0.05) in DS rats **(Figure 3.3)**.

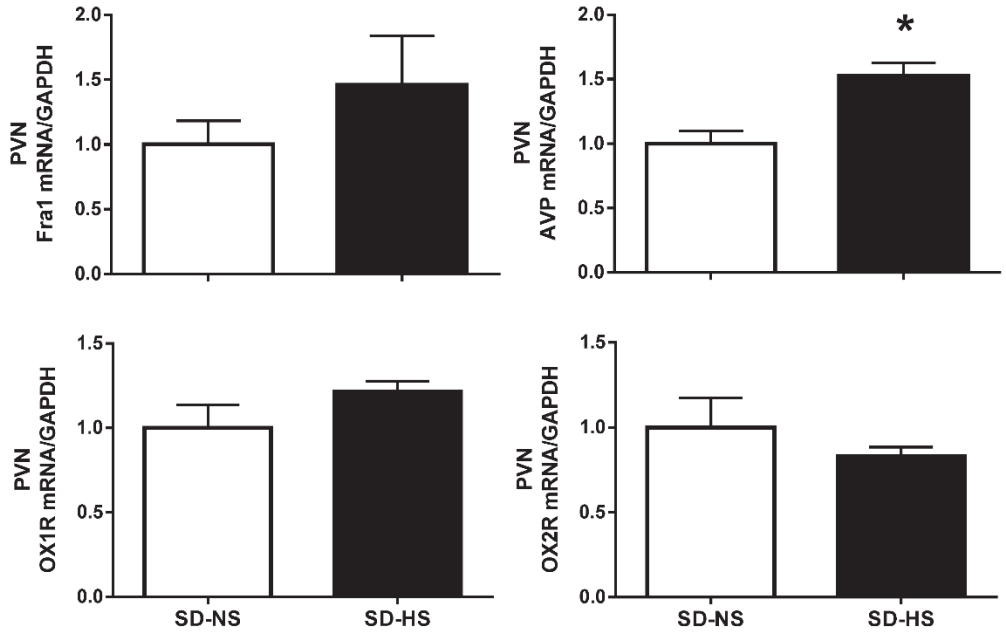


Figure 3.2 Real time PCR analysis of chronic neuronal activation marker Fra1, vasopressin (AVP), orexin receptor 1 (OX1R), and orexin receptor 2 (OX2R) mRNA expression levels in the paraventricular nucleus (PVN) between normal

salt (NS; 0.4% NaCl) and high salt (HS; 8% NaCl) Sprague Dawley (SD) rats. HS diet did not change Fra1, OX1R, or OX2R but significantly increased AVP PVN mRNA expression. The mRNA level in the control sample was assigned to be arbitrary unit (a.u) 1. *p≤0.05 vs. NS diet; n=3-6 each group.

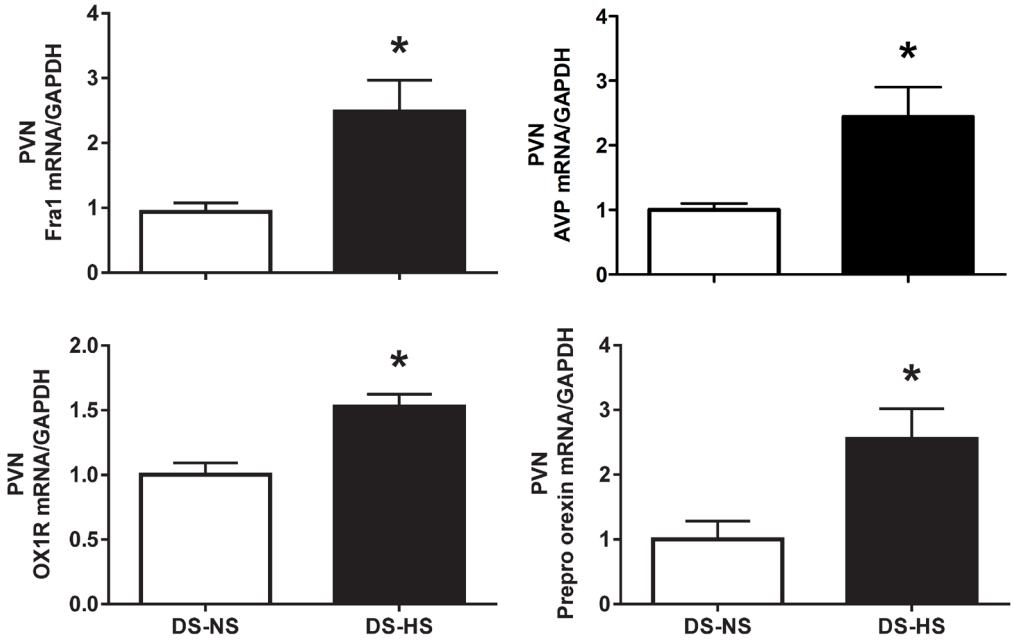


Figure 3.3 Real time PCR analysis of chronic neuronal activation marker Fra1, vasopressin (AVP), orexin receptor 1 (OX1R), and prepro orexin mRNA expression levels in the paraventricular nucleus (PVN) between normal salt (NS; 0.4% NaCl) and high salt (HS; 8% NaCl) Dahl salt sensitive (DS) rats. HS diet results in increases in mRNA levels of Fra1, AVP, OX1R, and prepro orexin in the PVN. The mRNA level in the control sample was assigned to be arbitrary unit (a.u) 1. *p≤0.05 vs. NS diet; n=5-7 each group.

3.3 ICV Orexin A induces increases in PVN AVP mRNA expression and Orexin A Induced Increases in AVP mRNA levels are Attenuated by OX1R Blockade in cultured Hypothalamic Neurons.

We hypothesized from the results from the above study that increased orexin receptor activation (OX1R or OX2R) in the PVN may be responsible for the enhanced PVN AVP levels in the HS intake DS rats. First In order to confirm orexin-A controls PVN AVP expression in vivo, male adult SD rats

received ICV injection of orexin-A (0.2 nmol). Three hours following injection, rats were euthanized and brain PVN tissues were punched out and received real time PCR analysis to determine AVP mRNA expression. The results showed that AVP mRNA levels were significantly increased 4.9-fold in orexin A injection rats (n=7) compared to vehicle control injected rats (P<0.05) **(Figure 3.4)**.

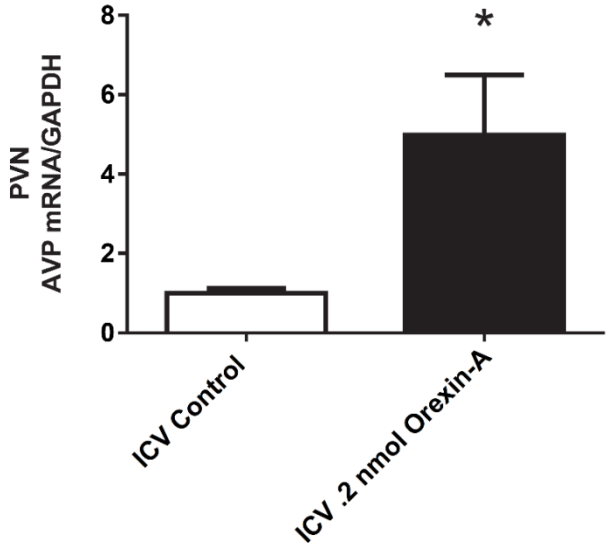


Figure 3.4: Real time PCR analysis of paraventricular nucleus (PVN) vasopressin (AVP) mRNA expression levels at three hours following acute intracerebroventricular (ICV) infusion of vehicle control (0.9% saline) and orexin A (0.2 nmol) using male adult Sprague Dawley (SD) rats. The mRNA level in the control sample was assigned to be arbitrary unit (a.u) 1. *p≤0.05 vs. vehicle control; n=7.

Next we determined which orexin receptor is responsible for the regulation of vasopressin (AVP) signaling. Primary neuronal cultures from the hypothalamus of new born SD rats containing the PVN were incubated with differing concentrations of orexin for six hours. Real time PCR was performed to assay mRNA levels of AVP. The results indicate that orexin treatment resulted in a dose dependent increase in AVP mRNA levels (100nM: 2.5-fold; 1μM; 4.8 fold; 10μM: 15.7-fold) (n=4) **(Figure 3.5 left)**. Next to determine the orexin receptor type responsible for inducing increased AVP expression, primary neuronal

cultures from the hypothalamus of new born SD rats were incubated with orexin alone or co-incubated with orexin A and the orexin receptor 1 antagonist (OX1Ra SB408124) or the orexin receptor 2 antagonist (OX2Ra TCS OX2 29) for 6 hours. Real time PCR was performed to measure AVP mRNA levels. The results showed that orexin (1μM) induced increases in AVP (2.7 fold) mRNA were attenuated by OX1Ra (1.2 fold) ($P<0.05$) but not OX2Ra (2.1 fold) (n=4) **(Figure 3.5 right)**.

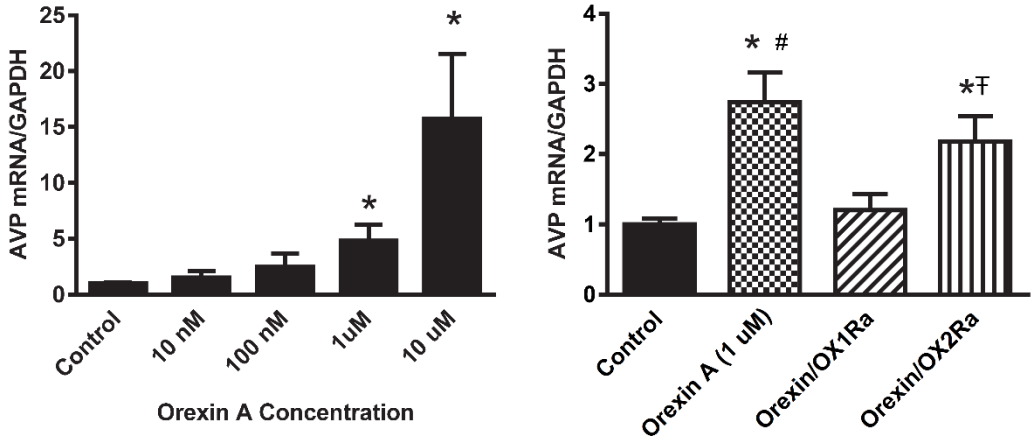


Figure 3.5 Left: Incubation of primary neuronal cultures from the hypothalamus of new born Sprague Dawley (SD) rats containing the paraventricular nucleus (PVN) with increasing concentrations of orexin for six hours, the neurons were then collected, RNA was purified, and real time PCR was performed to assay mRNA levels of AVP. *p ≤0.05 vs.control; n=4 each group. **Right:** Incubation of primary neuronal cultures from the hypothalamus of new born SD rats containing the PVN with orexin alone or co-incubated with an orexin receptor 1 antagonist (OX1Ra) SB408124 or orexin receptor 2 antagonist (OX2Ra) TCS OX2 29 for 6 hours. Real time PCR was performed to measure AVP mRNA levels. Orexin A induced increase in mRNA levels of vasopressin (AVP) are attenuated by OX1R but not OX2R blockade in hypothalamic neurons. The mRNA level in the control sample was assigned to be arbitrary unit (a.u) 1. *p ≤0.05 vs.control. #p≤0.05 vs. orexin/OX1Ra. ∓ P≤0.05 vs. orexin/OX1Ra treatment; n=4 each group.

3.4 Acute ICV Injection of Hypertonic Saline Increases PVN OX1R and AVP mRNA Levels in SD rats

The results from the above study suggest that orexin controls PVN AVP

expression through OX1R activation. This suggests that increased PVN OX1R activation, potentially via upregulation, may cause enhanced PVN AVP signaling in HS intake DS rats. It has been previously shown that CSF sodium concentration increases in DS rats on a HS diet t (64, 103). Therefore, we hypothesized that increased CSF sodium concentration in HS intake DS rats may cause PVN OX1R upregulation leading to enhanced PVN AVP signaling. It has previously been demonstrated that CSF sodium concentration of DS rats increased by 6mM after 6 days on an 8% HS diet (64). Therefore, we expect that after 4-6 weeks of 8% HS diet, CSF sodium concentration should increase by no less than 10mM. Assuming the entire CSF volume is 400 µl (142), ICV injection of 4 µmol NaCl should increase the CSF sodium concentration by 10mM. Male adult SD rats received ICV injection of hypertonic saline (4 µmol) in order to mimic increased CSF sodium concentration of DS rats on a HS diet for 5 weeks as used in our study. Three hours following injection PVN tissue was punched, RNA was extracted and reverse transcribed to cDNA and real time PCR was performed to determine PVN mRNA levels of OX1R, OX2R, and AVP. The results showed that OX1R (1.4 fold) but not OX2R (0.97 fold) PVN mRNA levels were significantly increased ($P<0.05$) in hypertonic saline injection rats compared to vehicle control injection rats. AVP mRNA levels were significantly increased (4.8-fold) in hypertonic saline injection rats (n=5) compared to vehicle control injected rats ($P<0.05$) **(Figure 3.6)**.

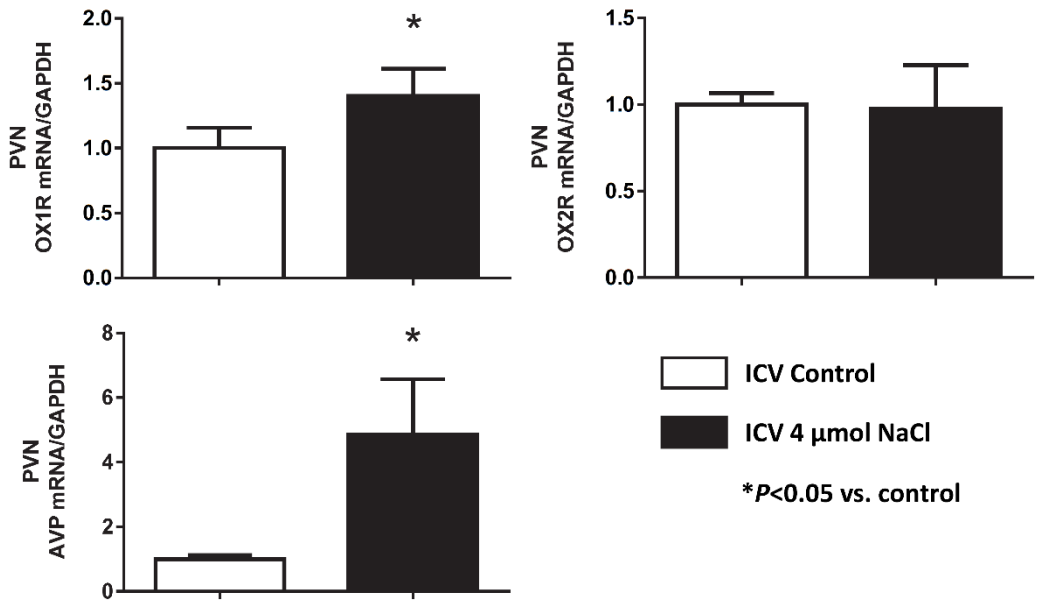


Figure 3.6 Real time PCR analysis of paraventricular nucleus (PVN) OX1R, OX2R, and vasopressin (AVP) mRNA expression levels at three hours following acute intracerebroventricular (ICV) infusion of vehicle control (0.9% saline) and hypertonic saline (4 µmol) using male adult Sprague Dawley (SD) rats. The mRNA level in the control sample was assigned to be arbitrary unit (a.u) 1. *p≤0.05 vs. vehicle control; n=4-7 each group.

3.5 OX1R Blockade in PVN Reduces Blood Pressure in HS Diet DS Rats

The results from the above studies indicate a HS diet increases PVN OX1R expression in DS rats, the OX1R mediates AVP signaling in the PVN, and increased CSF sodium concentration in HS intake DS rats may cause PVN OX1R upregulation leading to enhanced AVP signaling. Therefore, we hypothesized that increased OX1R activation in the PVN contributes to the maintenance of hypertension in HS intake DS rats. To test this hypothesis we recorded the MAP, SNA, and HR response to bilateral PVN microinjection of the OX1R antagonist SB408124 in anaesthetized DS rats fed a NS and HS diet for 5 weeks. OX1R antagonist SB408124 bilaterally injected into the PVN significantly decreased MAP in DS rats fed a HS diet compared to DS rats with NS intake. OX1R blockade increased SSNA and RSNA in NS and HS DS rats.

Figure 3.7 A-B (left panels) Shows an example of the SSNA, RSNA, and MAP responses to orexin receptor 1 antagonist (OX1Ra) SB408124 (30 pmol/50nl/side) into DS rats fed a NS diet (.4%). Maximum increases in SSNA and RSNA were (35.82±9%; n=5) and (17.4±10%; n=6) respectively. The maximum decrease in MAP was (-4±4 mmHg; n=6). **Figure 3.7 A-B** (right panels) Shows an example of the SSNA, RSNA, and MAP responses to SB408124 (30 pmol/50nl/side) into DS rats fed a HS diet (8%). Maximum increases in SSNA and RSNA were greater in HS rats compared to NS rats but failed to reach statistical significance (HS: 48.2±12.6%; n=5 vs. NS: 35.82±9%; n=5, P=0.2) and (HS: 26.7±7.5%; n=5 vs. NS: 17.4±10%; n=6, P=0.2) respectively. The maximum decrease in MAP was greater in HS rats compared to NS rats (HS: -16±5 mmHg; n=5 vs. NS: -4±4 mmHg; n=6, $P<0.05$). **Figure 3.8** shows PVN microinjection summary data. **Figure 3.9** shows a single representative PVN injection. It was found in four rats that dye was injected into the third ventricle therefore the results from these rats were not included for data analysis.

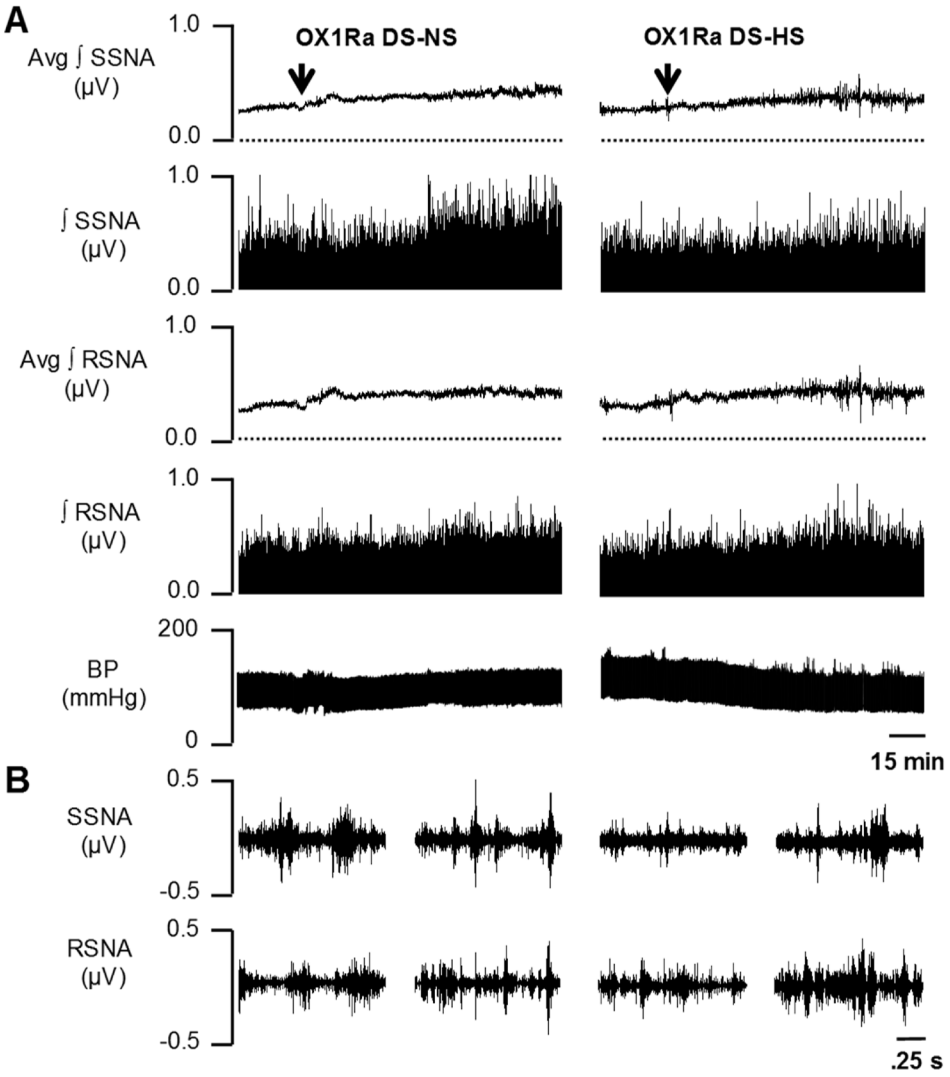


Figure 3.7 A: Representative traces showing splanchnic sympathetic nerve activity (SSNA), renal sympathetic nerve activity (RSNA), and mean arterial blood pressure (MAP) responses to bilateral PVN microinjections of Orexin receptor 1 antagonist (OX1Ra) SB408124 (30 pmol/50nl/side) into DS rats fed a normal salt diet (0.4%) left and DS rats fed a high salt diet (8%) right. **B:** Left column, 2.5-s specimen traces of SSNA (top) and RSNA (bottom) before injection of OX1Ra into the PVN and after microinjection of OX1Ra into the PVN of a DS rat on NS diet. Right column, 2.5-s specimen traces of SSNA (top) and RSNA (bottom) before injection of OX1Ra into the PVN and after microinjection of OX1Ra into the PVN of a DS rat on HS diet. PVN orexin receptor 1 blockade reduces blood pressure in DS rats with high salt intake.

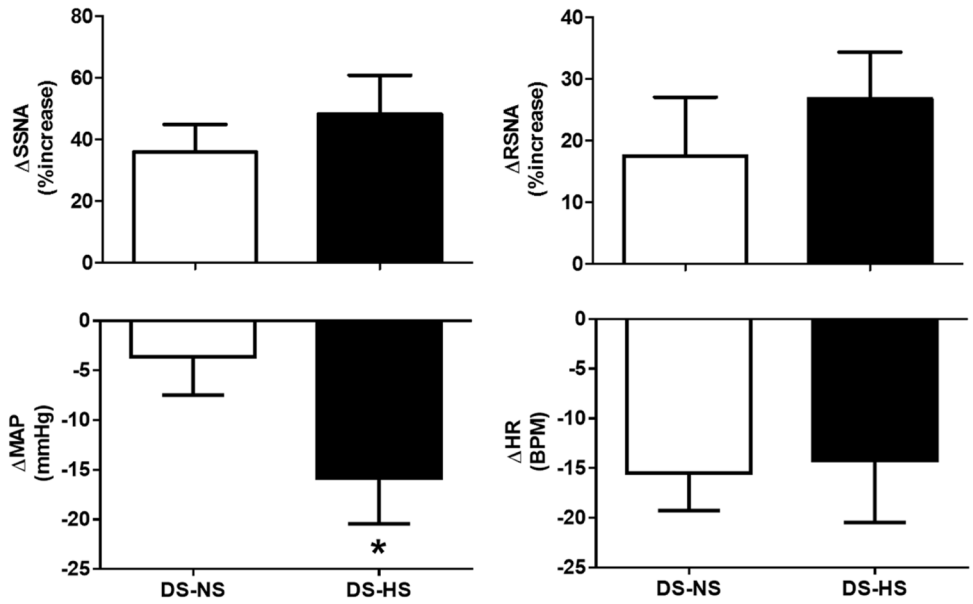
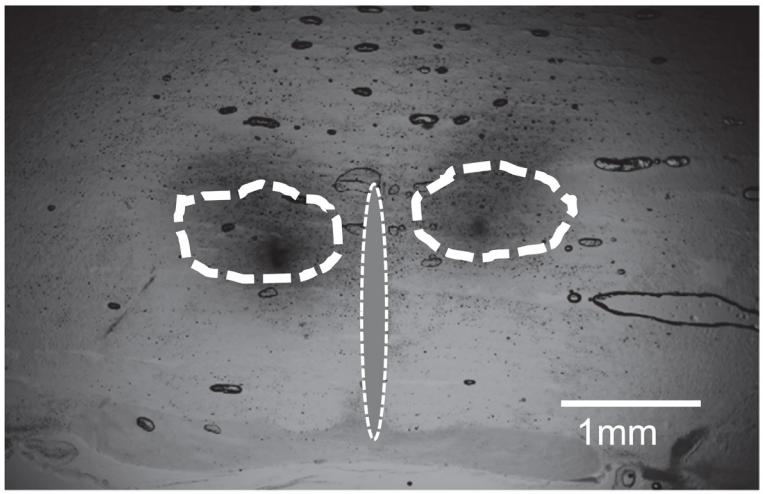


Figure 3.8 Summary data showing changes in splanchnic sympathetic nerve activity (SSNA), renal sympathetic nerve activity (RSNA), mean arterial pressure (MAP), and heart rate (HR) in response to paraventricular nucleus (PVN) bilateral microinjections of orexin receptor 1 antagonist SB408124 (30 pmol/50nl/side) in NS DS rats (n=5-6) and HS DS rats (n=5). *P<0.05 compared to NS.



Bregma -1.3 mm

Figure 3.9 Representation of a single injection (50nl) within the PVN.

Chapter 4. Discussion

Accumulating evidence suggests that the orexin system is involved in the central control of cardiovascular function and its hyperactivity in the brain contributes to hypertension (78, 79, 81, 152), however, the role of orexin in the SSHTN is not known. This study is the first to investigate the contribution of the orexin system to SSHTN. We report six findings. First MAP is increased in DS rats with HS intake. Second, Fra1, AVP, OX1R, and prepro orexin mRNA expression are upregulated in the PVN in DS rats with HS intake. Third, ICV administration of orexin A increased PVN AVP mRNA expression in SD rats. Fourth, Incubation of cultured hypothalamic neurons from SD rats containing the PVN with orexin increases AVP mRNA expression which is blocked by co-incubation with an OX1R antagonist. Fifth, ICV administration of hypertonic saline increase PVN OX1R and AVP mRNA levels in SD rats. Finally, PVN OX1R antagonist injection significantly reduces MAP in DS rats fed a HS diet compared to NS fed DS rats. Taken together, these findings indicate that increased OX1R activity in the PVN contributes to enhanced AVP signaling in DS rats on a HS diet which may contribute to the maintenance of Dahl SSHTN.

The PVN is a key site for the integration of autonomic and neuroendocrine function, and its function is essential to maintain elevated BP in Dahl SSHTN (49). Expression of orexin (15, 16, 105, 113, 126, 151) and its receptors (5, 18, 62, 83, 89, 144) are widespread in regions of the brain involved in blood pressure regulation including the PVN. Our study is novel in that it shows that mRNA expression levels of the orexin precursor prepro orexin and OX1R are higher in the PVN of DS rats on a HS diet compared to DS rats on a NS diet **(Figure 3.3)**. Our results also showed that DS rats on a HS diet had increased PVN AVP mRNA levels consistent with previous findings that plasma AVP is elevated in DS rats on a HS diet (90, 124, 146). We did not observe PVN mRNA expression of components of the orexin system in the

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The material contained in this chapter is in preparation for submission to a journal.

PVN to be changed in HS fed SD rats **(Figure 3.2)**. These results indicate that a HS diet may be linked to increased PVN orexin system activity in DS rats. Consistent with previous studies and as expected we found that 5 weeks HS 8% NaCl diet significantly increased MAP in DS rats but not SD rats **(Figure 3.1)**.

Based on these results we hypothesized that increased PVN orexin system activity may be responsible for increased AVP signaling in the PVN in HS DS rats. First, we sought to confirm that orexin A controls PVN AVP expression in vivo. ICV infusion of orexin A significantly augmented PVN AVP mRNA expression compared to vehicle control **(Figure 3.4)**. This is consistent with a previous study showing ICV injection of orexin A increased PVN AVP mRNA expression in conscious rats (1) and ICV administration of orexin A significantly increased plasma AVP levels in conscious rabbits after 90 minutes (91). Next, we sought to determine the receptor type orexin A may control AVP signaling in the PVN. We performed this study in vitro using cultured neurons from the hypothalamus containing the PVN from newborn SD rats. Incubation of cultured brain neurons with orexin A resulted in a dose dependent increase in AVP mRNA expression **(Figure 3.5)**. Co-incubation with an OX1R but not with an OX2R antagonist attenuated this orexin induced AVP mRNA expression response **(Figure 3.5)**. Taken together these results indicate that activation of the OX1R expressed on PVN neurons is likely responsible for orexin induced AVP mRNA expression. This suggest that increased PVN OX1R activation potentially via upregulation contributes to enhanced PVN AVP signaling of DS rats on a HS diet.

DS rats on a HS diet are associated with increased CSF sodium concentration (64, 103) which precedes hypertension (64). It was found the CSF sodium concentration of DS rats but not Dahl salt resistant rats increased by 6mM after 6 days on an 8% HS diet preceding hypertension (64). Therefore, we hypothesized that increased CSF sodium concentration in HS intake DS rats may cause PVN OX1R upregulation leading to enhanced PVN AVP signaling. ICV infusion of hypertonic saline in SD rats was performed to mimic

an increase in CSF sodium concentration to levels observed in HS fed DS rats. The results showed that ICV infusion of 4 µmol NaCl significantly increased PVN OX1R and AVP mRNA expression **(Figure 3.6)** which suggests that increased CSF sodium concentration, a main phenotype component of DS rats on a HS diet, may cause PVN OX1R upregulation leading to enhanced AVP signaling. There have been no studies that have directly determined if orexin producing neurons are sensitive to changes in osmolality. It has previously been shown that preopro-orexin mRNA is upregulated In the hypothalamus of 48 hour water deprived rats indicating orexin producing neurons may be sensitive to changes in osmolality (75). Also the lamina terminalis which is the location of the primary central osmoreceptors is reported to have efferent projections to the lateral hypothalamus where orexin producing cell bodies are located (58, 65) which may explain why orexin neurons would be sensitive to changes in body fluid osmolality. Interestingly it has been hypothesized that orexin neurons may be a link between the lamina terminalis sensing of perturbations in body fluid homeostasis and activating motivation and reward systems in the mesolimbic system (dopaminergic projection from the ventral tegmental area to the Nucleus accumbens) to effect salt and water appetite motivated behavior in order to restore body fluid homeostasis (65).

Finally, since we found DS rats on HS diet displayed increased PVN mRNA levels of OX1R, OX1R regulates PVN AVP expression, and increased CSF sodium concentration in HS intake DS rats may cause PVN OX1R upregulation leading to enhanced AVP signaling, we determined if increased OX1R activation in the PVN was implicated in HS induced hypertension in DS rats. Microinjection of The OX1R antagonist SB408124 into the PVN elicited a significantly greater reduction in MAP in HS DS rats compared to NS DS rats **(Figure 3.8)**. This result suggests that increased PVN OX1R activation contributes to the maintenance of HS induced hypertension in DS rats. This is likely through both increased orexin release into the PVN or increased PVN OX1R expression since both prepro orexin and OX1R mRNA expression were upregulated in the PVN of HS DS rats. PVN OX1R blockade also increased

both SSNA and RSNA in NS and HS rats. Since this experiment was performed in baroreceptor intact animals this SNA response is likely driven by the arterial baroreflex mechanism. It should be noted that we did not observe a parallel reduction in SNA along with the reduction in MAP therefore, it is plausible that OX1R expressed on magnocellular PVN neurons is responsible for AVP expression and increased OX1R activation results in greater AVP expression and possibly release in DS rats on HS diet contributing to the maintenance of hypertension. Evidence indicates orexin A may bind the OX1R and OX2R on presynaptic or interneuron glutamatergic PVN neurons and increases glutamate release to magnocellular PVN neurons (47). Also orexin A may bind OX1R or OX2R on magnocellular PVN neurons directly and positively modulates glutamate receptor activity (47). Although the contribution of AVP to Dahl SSHTN is still controversial, recently it was demonstrated that oral administration of the dual AVP receptor antagonist RWJ-676070 produced a transient decrease in MAP in hypertensive DS rats on a HS diet (53) contributing to evidence that AVP does contribute to Dahl SSHTN. However, we still cannot ignore the possibility that increased OX1R activation on parvocellular PVN neurons may be responsible for increased sympathetic tone and elevated BP in HS DS rats. Performing this same experiment with rats that have received sino-aortic denervation or severing of the baroreceptor afferents may clarify whether the mechanism is due to activation of OX1R on magnocellular or parvocellular neurons. We hypothesize that if increased OX1R activation on parvocellular PVN neurons is responsible for enhanced neuronal activity and elevated SNA contributing to hypertension of HS fed DS rats then OX1R blockade would result in a reduction in both SNA and MAP in sino-aortic denervated rats.

4.1 Summary and Conclusion

To summarize, the current study showed that a HS diet increased MAP in DS but not SD rats. DS rats on a HS diet have increased PVN mRNA expression of Fra1, AVP, orexin precursor, and OX1R indicating the PVN orexin system may be overactive in hypertensive HS DS rats which display

enhanced PVN AVP expression. HS intake did not alter PVN mRNA expression of Fra1, OX1R, or OX2R but increased PVN AVP mRNA expression in HS SD rats. ICV injection of orexin A in SD rats significantly increased PVN mRNA expression of AVP. In vitro studies showed activation of OX1R expressed on hypothalamic neurons mediates AVP expression. ICV infusion of hypertonic saline in SD rats increased PVN OX1R and AVP mRNA expression. PVN OX1R blockade significantly reduced MAP in DS rats on a HS diet compared to DS rats on a NS diet. In conclusion, these findings indicate that increased OX1R activity in the PVN contributes to enhanced AVP signaling in DS rats on a HS diet which may contribute to the maintenance of Dahl SSHTN.

4.2 Perspectives

Individuals with hypertension are at an increased risk for cardiovascular disease, the leading global cause of death. Increased dietary salt intake is a primary contributor to essential hypertension. Therefore, it is of great interest to elucidate how salt effects the blood pressure regulatory mechanisms in this salt sensitive population in order to further treatment possibilities to reduce the number of deaths caused by cardiovascular diseases. The present study identifies a novel mechanism were the orexin system may contribute to SSHTN by enhancing AVP signaling.

4.3 Limitations and Future Studies

There are several limitations that should be acknowledged for this study. First, PVN brain tissue punch for real-time PCR analysis of mRNA expression is not exact. Due to the small size of the PVN we acknowledge the possibility that other surrounding nuclei are included. Additionally, the PVN is composed of magnocellular AVP producing and pre sympathetic parvocellular neurons. Therefore, we are unable to determine whether results from real-time PCR analysis of mRNA expression reflect a specific neuron type. It is also important to note that animals used for sympathetic nerve recording experiments use an anaesthetized preparation. This may alter cardiovascular responses to microinjection of drugs used in this study (46). A major limitation in this study is

the use of baroreflex intact animals. The baroreflex senses and corrects acute changes in blood pressure through modulation of SNA. Therefore, responses to drugs injected into the PVN may be compensated for by reflex modulation of SNA making interpretation of results difficult. Future studies will include sino-aortic denervated animals in order to remove the baroreflex response. Ongoing studies in our lab are examining the role of the PVN orexin system in augmented sympathetic outflow in the Dahl salt sensitive hypertension model.

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Appendix A. Raw Data

Table A.1 Raw data for Conscious Mean Arterial Blood Pressure for Sprague Dawley and Dahl Salt Sensitive Rats on Normal Salt and High Salt diet.

MAP (mmHg)			
SD NS	**SD HS**	**DSNS**	**DS HS**
104	113	114	179
103	110	106	173
104	110	112	150
105	101	113	153
110	108	112	159
106	107	–	140

Table A.2 Raw data for PVN Fra1 mRNA Expression for Sprague Dawley Rats on Normal Salt and High Salt Diet.

Fra1 mRNA expression normalized to GAPDH mRNA (a.u.)	
SD NS	**SD HS**
0.6	0.66
0.77	2.34
1.76	1.8
0.61	1.02
0.97	–
1.25	–

Table A.3 Raw data for PVN Arginine Vasopressin mRNA Expression for Sprague Dawley and Dahl Salt Sensitive Rats on Normal Salt and High Salt Diet.

AVP mRNA expression normalized to GAPDH mRNA (a.u.)	
SD NS	**SD HS**
0.61	1.63
1.21	1.61
1.17	1.33
1.18	–
0.97	–
0.83	–

Table A.4 Raw data for PVN Orexin Receptor 1 mRNA Expression for Sprague Dawley Rats on Normal Salt and High Salt Diet.

OX1R mRNA expression normalized to GAPDH mRNA (a.u.)	
SD NS	**SD HS**
0.89	1.16
1.00	1.08
1.37	1.29
0.72	1.45
–	1.05
–	1.25

Table A.5 Raw data for PVN Orexin Receptor 2 mRNA Expression for Sprague Dawley Rats on Normal Salt and High Salt Diet.

OX2R mRNA expression normalized to GAPDH mRNA (a.u.)	
SD NS	**SD HS**
0.75	0.68
0.82	0.8
1.52	0.83
0.89	0.97

Table A.6 Raw data for PVN Fra1 mRNA Expression for Dahl Salt Sensitive Rats on Normal Salt and High Salt Diet.

Fra1 mRNA expression normalized to GAPDH mRNA (a.u.)	
DS NS	**DS HS**
0.40	3.13
1.22	4.55
0.97	2.11
1.23	1.4
1.13	1.54
0.65	2.15

Table A.7 Raw data for PVN Arginine Vasopressin mRNA Expression for Dahl Salt Sensitive Rats on Normal Salt and High Salt Diet.

AVP mRNA Expression Normalized to GAPDH mRNA (a.u.)	
DS NS	**DS HS**
1.12	1.89
1.16	1.75
0.70	4.69
0.82	1.82
1.17	2.09
–	2.37

Table A.8 Raw data for PVN Prepro Orexin mRNA Expression for Dahl Salt Sensitive Rats on Normal Salt and High Salt Diet.

Prepro Orexin mRNA Expression Normalized to GAPDH mRNA (a.u.)	
DS NS	**DS HS**
2.34	3.16
0.48	4.45
0.16	3.76
1.66	1.52
0.82	1.61
0.94	1.9
0.56	1.42

Table A.9 Raw data for PVN Orexin Receptor 1 mRNA Expression for Dahl Salt Sensitive Rats on Normal Salt and High Salt Diet.

OX1R mRNA Expression Normalized to GAPDH mRNA (a.u.)	
DS NS	**DS HS**
0.79	1.51
1.00	1.38
1.20	1.21
1.35	2.03
0.61	1.51
0.97	1.62
1.05	1.41
–	0.99
–	0.68

Table A.10 Raw data for PVN Arginine Vasopressin mRNA Expression Response to Intracerebroventricular injections of Isotonic saline and .2 nmol Orexin A in Sprague Dawley Rats.

AVP mRNA expression normalized to GAPDH mRNA (a.u.)	
Control	**.2 nmol Orexin A**
1.00	13.41
1.41	4.83
0.59	4.17
0.71	5.98
1.17	1.65
1.35	2.06
0.77	2.73

Table A.11 Raw data for Arginine Vasopressin mRNA Expression Response to 10 nM, 100 nM, 1 µM, and 10 µM Orexin treated Cultured Hypothalamic Neurons.

AVP mRNA Expression Normalized to GAPDH mRNA (a.u.)				
Control	**10 nM Orexin**	**100 nM Orexin**	**1 µM Orexin**	**10 µM Orexin**
1.37	1.76	4.12	6.25	14.22
0.63	0.96	1.66	5.02	22.69
0.83	1.09	2.56	2.79	8.72
1.17	2.28	1.53	5.17	17.26

Table A.12 Raw data for Arginine Vasopressin mRNA Expression Response to 1 µM Orexin, Orexin co-incubated with Orexin Receptor 1 Antagonist, Orexin co-incubated with Orexin Receptor 2 Antagonist treated Cultured Hypothalamic Neurons.

AVP mRNA expression normalized to GAPDH mRNA (a.u.)			
Control	**1 µM Orexin**	**Orexin/OX1Ra**	**Orexin/OX2Ra**
1.10	1.96	0.65	1.58
0.90	2.22	1.11	2.60
0.81	3.82	1.73	2.98
1.19	2.98	1.32	1.56

Table A.13 Raw data for PVN Orexin Receptor 1 mRNA Expression Response to Intracerebroventricular injections of Isotonic saline and 4µmol NaCl in Sprague Dawley Rats.

OX1R mRNA expression normalized to GAPDH mRNA (a.u.)	
Control	**4 µmol NaCl**
1.55	1.23
0.45	1.53
1.08	1.03
0.91	1.52
0.46	1.65
1.23	1.37

1.29	1.44

Table A.14 Raw data for PVN Orexin Receptor 2 mRNA Expression Response to Intracerebroventricular injections of Isotonic saline and 4µmol NaCl in Sprague Dawley Rats.

OX2R mRNA expression normalized to GAPDH mRNA (a.u.)	
Control	**4 µmol NaCl**
1.13	1.10
0.95	0.60
0.83	1.08
1.08	1.11

Table A.15 Raw data for PVN Arginine Vasopressin mRNA Expression Response to Intracerebroventricular injections of Isotonic saline and 4µmol NaCl in Sprague Dawley Rats.

AVP mRNA expression normalized to GAPDH mRNA (a.u.)	
Control	**4 µmol NaCl**
1.00	11.13
1.41	5.73
0.59	–
0.71	1.99
1.17	1.84
1.35	3.57
0.77	–

Table A.16 Raw data for Splanchnic and Renal Sympathetic Nerve Activity response to Bilateral 30 pmol SB408124 PVN injections in Dahl Salt Sensitive Rats on Normal Salt diet.

	SSNA				RSNA			
Rat	**Base (µV)**	**Max (µV)**	**%Change**	**Noise (µV)**	**Base (µV)**	**Max (µV)**	**%Change**	**Noise (µV)**
1	0.046	0.057	37.039	0.016	0.018	0.023	45.633	0.008
2	0.040	0.042	8.653	0.023	0.017	0.018	8.264	0.005
3	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	–	–
5	–	–	–	–	0.015	0.017	21.402	0.005
6	0.022	0.027	51.236	0.013	0.009	0.008	-23.523	0.005

7	0.026	0.034	58.240	0.014	0.042	0.05	29.229	0.017
8	0.022	0.025	23.907	0.010	0.028	0.031	23.859	0.017

Table A.17 Raw data for Mean Arterial Pressure and Heart Rate response to Bilateral 30 pmol SB408124 PVN injections in Dahl Salt Sensitive Rats on Normal Salt diet.

	MAP			HR		
Rat	Base (mmHg)	Max (mmHg)	Delta (mmHg)	Base (mmHg)	Max (mmHg)	Delta (mmHg)
1	110	111	1	394	375	-19
2	122	118	-4	363	366	3
3	–	–	–	–	–	–
4	–	–	–	–	–	–
5	102	106	4	378	360	-18
6	121	103	-18	385	364	-21
7	112	118	6	416	399	-17
8	106	95	-11	386	365	-21

Table A.18 Raw data for Splanchnic and Renal Sympathetic Nerve Activity response to Bilateral 30 pmol SB408124 PVN injections in Dahl Salt Sensitive Rats on High Salt diet.

	SSNA				RSNA			
Rat	Base (μV)	Max (μV)	%Change	Noise (μV)	Base (μV)	Max (μV)	%Change	Noise (μV)
1	–	–	–	–	–	–	–	–
2	–	–	–	–	0.059	0.075	36.191	0.015
3	–	–	–	–	–	–	–	–
4	0.049	0.066	88.610	0.031	0.020	0.022	15.927	0.007
5	–	–	–	–	–	–	–	–
6	0.041	0.048	33.406	0.019	0.021	0.024	33.322	0.012
7	0.050	0.059	26.430	0.017	0.030	0.031	3.222	0.016
8	0.031	0.042	66.841	0.015	0.041	0.054	45.275	0.011

Table A.19 Raw data for Mean Arterial Pressure and Heart Rate response to Bilateral 30 pmol SB408124 PVN injections in Dahl Salt Sensitive Rats on High Salt diet.

	MAP			HR		
Rat	Base (mmHg)	Max (mmHg)	Delta (mmHg)	Base (mmHg)	Max (mmHg)	Delta (mmHg)
1	–	–	–	–	–	–
2	148	119	-29	327	326	-1

3	–	–	–	–	–	–
4	152	128	-24	365	342	-23
5	–	–	–	–	–	–
6	132	128	-4	345	348	3
7	144	133	-11	425	400	-25
8	146	135	-11	346	321	-25

Appendix B. Summary Statistics

Table B.1 Average Conscious Mean Arterial Blood Pressure for Sprague Dawley and Dahl Salt Sensitive Rats on Normal Salt and High Salt diet.

	SD NS	SD HS	DS NS	DS HS
Number of values	6	6	5	6
Mean	105.3	108.2	111.40	159.00
Std. Deviation	2.50	4.07	3.13	14.66
Std. Error of Mean	1.02	1.66	1.40	5.98
Lower 95% CI of mean	102.7	103.9	107.5	143.6
Upper 95% CI of mean	108.0	112.4	115.3	174.4

Table B.2 Average PVN Fra1 mRNA Expression for Sprague Dawley and Dahl Salt Sensitive Rats on Normal Salt and High Salt Diet.

	SD NS	SD HS	DS NS	DS HS
Number of values	6	4	6	6
Mean	1.00	1.46	0.93	2.48
Std. Deviation	0.44	0.75	0.33	1.18
Std. Error of Mean	0.18	0.37	0.13	0.48
Lower 95% CI of mean	0.53	0.25	0.58	1.24
Upper 95% CI of mean	1.46	2.66	1.29	3.72

Table B.3 Average PVN Arginine Vasopressin mRNA Expression for Sprague Dawley and Dahl Salt Sensitive Rats on Normal Salt and High Salt Diet.

	SD NS	SD HS	DS NS	DS HS
Number of values	6	3	5	6
Mean	1.00	1.52	1.00	2.44
Std. Deviation	0.24	0.16	0.21	1.12
Std. Error of Mean	0.09	0.09	0.09	0.46
Lower 95% CI of mean	0.74	1.1	0.73	1.25
Upper 95% CI of mean	1.25	1.94	1.26	3.62

Table B.4 Average PVN Prepro Orexin mRNA Expression for Dahl Salt Sensitive Rats on Normal Salt and High Salt Diet.

	DS NS	DS HS
Number of values	7	7
Mean	1.00	2.52
Std. Deviation	0.75	1.23
Std. Error of Mean	0.28	0.46
Lower 95% CI of mean	0.29	1.41
Upper 95% CI of mean	1.70	3.69

Table B.5 Average PVN Orexin Receptor 1 mRNA Expression for Sprague Dawley and Dahl Salt Sensitive Rats on Normal Salt and High Salt Diet.

	SD NS	SD HS	DS NS	DS HS
Number of values	4	6	7	7
Mean	1.00	1.21	1.00	1.52
Std. Deviation	0.27	0.14	0.24	0.25
Std. Error of Mean	0.13	0.06	0.09	0.09
Lower 95% CI of mean	0.56	1.06	0.77	1.29
Upper 95% CI of mean	1.43	1.37	1.22	1.76

Table B.6 Average PVN Orexin Receptor 2 mRNA Expression for Sprague Dawley Rats on Normal Salt and High Salt Diet.

	SD NS	SD HS
Number of values	4	6
Mean	1.00	0.82
Std. Deviation	0.35	0.13
Std. Error of Mean	0.17	0.05
Lower 95% CI of mean	0.44	0.68
Upper 95% CI of mean	1.55	0.97

Table B.7 Average PVN Arginine Vasopressin mRNA Expression Response to Intracerebroventricular injections of Isotonic saline and .2 nmol Orexin A in Sprague Dawley Rats.

	Control	.2 nmol Orexin A
Number of values	7	7
Mean	1.00	4.97
Std. Deviation	0.32	4.02
Std. Error of Mean	0.12	1.52
Lower 95% CI of mean	0.70	1.24
Upper 95% CI of mean	1.29	8.70

Table B.8 Average Arginine Vasopressin mRNA Expression Response to 10 nM, 100 nM, 1 μM, and 10 μM Orexin A treated Cultured Hypothalamic Neurons.

	Control	10 nM Orexin	100 nM Orexin	1 μM Orexin	10 μM Orexin
Number of values	4	4	4	4	4
Mean	1.00	1.52	2.46	4.80	15.72
Std. Deviation	0.33	0.61	1.19	1.45	5.83
Std. Error of Mean	0.16	0.30	0.59	0.72	2.91
Lower 95% CI of mean	0.47	0.54	0.56	2.49	6.43
Upper 95% CI of mean	1.52	2.5	4.36	7.11	25.01

Table B.9 Average Arginine Vasopressin mRNA Expression Response to 1 μM Orexin, Orexin co-incubated with Orexin Receptor 1 Antagonist, Orexin co-incubated with Orexin Receptor 2 Antagonist

	Control	1 μM Orexin	Orexin/ OX1Ra	Orexin/ OX2Ra
Number of values	4	4	4	4
Mean	1.00	2.74	1.20	2.18
Std. Deviation	0.17	0.83	0.44	0.72
Std. Error of Mean	0.08	0.41	0.22	0.36
Lower 95% CI of mean	0.72	1.41	0.48	1.03

Upper 95% CI of mean	1.27	4.07	1.91	3.32

Table B.10 Average PVN Orexin Receptor 1 mRNA Expression Response to Intracerebroventricular injections of Isotonic saline and 4µmol NaCl in Sprague Dawley Rats.

	Control	**4 µmol NaCl**
Number of values	7	7
Mean	1.00	1.40
Std. Deviation	0.41	0.21
Std. Error of Mean	0.15	0.07
Lower 95% CI of mean	0.61	1.20
Upper 95% CI of mean	1.38	1.59

Table B.11 Average PVN Orexin Receptor 2 mRNA Expression Response to Intracerebroventricular injections of Isotonic saline and 4µmol NaCl in Sprague Dawley Rats.

	Control	**4 µmol NaCl**
Number of values	4	4
Mean	1.00	0.97
Std. Deviation	0.13	0.25
Std. Error of Mean	0.06	0.12
Lower 95% CI of mean	0.78	0.57
Upper 95% CI of mean	1.21	1.37

Table B.12 Average PVN Arginine Vasopressin mRNA Expression Response to Intracerebroventricular injections of Isotonic saline and 4µmol NaCl in Sprague Dawley Rats.

	Control	4 µmol NaCl
Number of values	7	5
Mean	1.00	4.85
Std. Deviation	0.32	3.84
Std. Error of Mean	0.12	1.71
Lower 95% CI of mean	0.70	0.08
Upper 95% CI of mean	1.29	9.62

Table B.13 Mean splanchnic sympathetic nerve activity % change from baseline following PVN microinjection of OX1R antagonist SB408124 in Normal Salt and High Salt Dahl Salt Sensitive rats.

	SB408124 DS NS	SB408124 DS HS
Number of values	5	5
Mean	35.82	48.20
Std. Deviation	20.14	28.19
Std. Error of Mean	9.00	12.61
Lower 95% CI of mean	10.81	13.19
Upper 95% CI of mean	60.82	83.21

Table B.14 Mean renal sympathetic nerve activity % change from baseline following PVN microinjection of OX1R antagonist SB408124 in Normal Salt and High Salt Dahl Salt Sensitive rats.

	SB408124 DS NS	SB408124 DS HS
Number of values	6	5
Mean	17.48	26.79
Std. Deviation	23.46	16.93
Std. Error of Mean	9.57	7.57

Lower 95% CI of mean	-7.14	5.76
Upper 95% CI of mean	42.10	47.81

Table B.15 Mean change from baseline for mean arterial pressure (mmHg) following PVN microinjection of OX1R antagonist SB408124 in Normal Salt and High Salt Dahl Salt Sensitive rats.

	SB408124 DS NS	**SB408124 DS HS**
Number of values	6	5
Mean	-3.66	-15.80
Std. Deviation	9.30	10.33
Std. Error of Mean	3.80	4.62
Lower 95% CI of mean	-13.44	-28.63
Upper 95% CI of mean	6.10	-2.97

Table B.16 Mean change from baseline for heart rate (beats/minute) following PVN microinjection of OX1R antagonist SB408124 in Normal Salt and High Salt Dahl Salt Sensitive rats.

	SB408124 DS NS	**SB408124 DS HS**
Number of values	6	5
Mean	-15.50	-14.20
Std. Deviation	9.20	13.97
Std. Error of Mean	3.75	6.24
Lower 95% CI of mean	-25.16	-31.55
Upper 95% CI of mean	-5.84	3.14

Table B.17 Unpaired t-test comparing MAP and PVN mRNA expression of Fra1, Arginine Vasopressin, Orexin Receptor 1, and Orexin Receptor 2 between Normal Salt and High Salt Sprague Dawley rats.

	Fra1 mRNA	AVP mRNA	OX1R mRNA	OX2R mRNA	MAP
Group 1	SD NS	SD NS	SD NS	SD NS	SD NS
vs.	vs	vs	vs	vs	vs
Group 2	SD HS	SD HS	SD HS	SD HS	SD HS
Unpaired t test					
P value	0.2573	0.0121	0.1411	0.3033	0.177
P value summary	ns	*	ns	ns	ns
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed	Two-tailed	Two-tailed
t, df	t=1.220 df=8	t=3.357 df=7	t=1.633 df=8	t=1.100 df=8	t=1.452 df=10

Table B.18 Unpaired t-test comparing MAP PVN mRNA expression of Fra1, Arginine Vasopressin, Prepro Orexin, and Orexin Receptor 1 between Normal Salt and High Salt Dahl Salt Sensitive Rats.

	Fra1 mRNA	AVP mRNA	Prepro Orexin mRNA	OX1R mRNA	MAP
Group 1	DS NS	DS NS	DS NS	DS NS	DS NS
vs.	vs	vs	vs	vs	vs
Group 2	DS HS	DS HS	DS HS	DS HS	DS HS
Unpaired t test					
P value	0.0117	0.0210	0.0148	0.0019	<0.0001
P value summary	*	*	*	**	****
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed	Two-tailed	Two-tailed
t, df	t=3.077 df=10	t=2.791 df=9	t=2.842 df=12	t=3.959 df=12	t=7.068 df=9

Table B.19 Unpaired t-test comparing PVN Arginine Vasopressin mRNA Expression Response to Intracerebroventricular injections of control vs. 2 nmol Orexin A in Sprague Dawley Rats.

	AVP mRNA
Group 1	Control
vs.	vs
Group 2	.2 nmol Orexin A
Unpaired t test	
P value	0.0232
P value summary	*
One- or two-tailed P value?	Two-tailed
t, df	t=2.602 df=12

Table B.20 Unpaired t-test comparing Arginine Vasopressin mRNA Expression Response to Control vs. 10nM orexin, 100 nM orexin, 1 μM Orexin, and 10 μM Orexin treatment in cultured hypothalamic neurons.

	AVP mRNA	AVP mRNA	AVP mRNA	AVP mRNA
Group 1	Control	Control	Control	Control
vs.	vs	vs	vs	vs
Group 2	10 nM Orexin	100 nM Orexin	1 μM Orexin	10 μM Orexin
Unpaired t test				
P value	0.1855	0.0555	0.0022	0.0024
P value summary	ns	ns	**	**
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed	Two-tailed
t, df	t=1.495 df=6	t=2.370 df=6	t=5.111 df=6	t=5.037 df=6

Table B.21 One way anova with post hoc Newman-Keuls test comparing Arginine Vasopressin mRNA Expression Response between control, 1 μM Orexin, Orexin co-incubated with Orexin Receptor 1 Antagonist, Orexin co-incubated with Orexin Receptor 2 Antagonist treated hypothalamic neurons.

ANOVA summary	
F	7.458
P value	0.0044
R square	0.6509

ANOVA table	SS	DF	MS
Treatment (between columns)	8.132	3	2.711
Residual (within columns)	4.362	12	0.3635
Total	12.49	15	

Newman-Keuls	Mean Diff.	Significant ?	Summary
Control vs 1 µM Orexin	-1.745	Yes	**
Control vs Orexin/ OX2Ra	-1.18	Yes	*
Control vs Orexin/ OX1Ra	-0.202	No	ns
Orexin/OX1Ra vs 1 µM Orexin	-1.543	Yes	**
Orexin/OX1Ra vs Orexin/OX2Ra	-0.977	Yes	*
Orexin/OX2Ra vs 1 µM Orexin	-0.565	No	ns

Table B.22 Unpaired t-test comparing Orexin Receptor 1, Orexin Receptor 2, and Arginine Vasopressin mRNA response between intracerebroventricular injections of isotonic saline vs. 4 µmol NaCl in Sprague Dawley rats.

	OX1R mRNA	OX2R mRNA	AVP mRNA
Group 1	Control	Control	Control
vs.	vs	vs	vs
Group 2	4 µmol NaCl	4 µmol NaCl	4 µmol NaCl
Unpaired t test			
P value	0.0433	0.87	0.0226
P value summary	*	ns	*
One- or two-tailed P value?	Two-tailed	Two-tailed	Two-tailed
t, df	t=2.259 df=12	t=0.1663, df=6	t=2.694 df=10

Table B.23 Unpaired t-test comparing splanchnic sympathetic nerve activity % change, renal sympathetic nerve activity % change, mean arterial pressure, and heart rate change from baseline following PVN microinjection of OX1R antagonist SB408124 in Normal Salt and High Salt Dahl Salt Sensitive rats.

	SSNA	RSNA	MAP	HR
Group 1	SB408124 DS NS	SB408124 DS NS	SB408124 DS NS	SB408124 DS NS
vs.	vs	vs	vs	vs
Group 2	SB408124 DS HS	SB408124 DS HS	SB408124 DS HS	SB408124 DS HS
Unpaired t test				
P value	0.2236	0.2395	0.0353	0.4284
P value summary	ns	ns	*	ns
One- or two-tailed P value?	One-tailed	One-tailed	One-tailed	One-tailed
t, df	t=0.7992 df=8	t=0.7387 df=9	t=2.050 df=9	t=0.1856 df=9